PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 08 January 1998 (08.01.98)	
International application No. PCT/GB97/01412	**Applicant's or agent's file reference** P17156WO
International filing date (day/month/year) 23 May 1997 (23.05.97)	**Priority date** (day/month/year) 24 May 1996 (24.05.96)
Applicant NERI, Dario et al	

1. The designated Office is hereby notified of its election made:

   [X] in the demand filed with the International Preliminary Examining Authority on:

   11 December 1997 (11.12.97)

   [ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

   [ ] was not

   made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

M. Abidine

Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

NOTARBARTOLO & GERVASI SRL
Via Savoia 82
I-00198 Rome
ITALIE

Date of mailing (day/month/year)
03 February 1998 (03.02.98)

Applicant's or agent's file reference
P17156WO

IMPORTANT NOTIFICATION

International application No.
PCT/GB97/01412

International filing date (day/month/year)
23 May 1997 (23.05.97)

1. The following indications appeared on record concerning:
☐ the applicant ☐ the inventor ☒ the agent ☐ the common representative

Name and Address
HOWARD, Paul, Nicholas
Carpmaels & Ransford
43 Bloomsbury Square
London WC1A 2RA
United Kingdom

State of Nationality | State of Residence

Telephone No.
0171 242 8692

Facsimile No.
0171 405 4166

Teleprinter No.
267209

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:
☒ the person ☐ the name ☒ the address ☐ the nationality ☐ the residence

Name and Address
NOTARBARTOLO & GERVASI SRL
Via Savoia 82
I-00198 Rome
Italy

State of Nationality | State of Residence

Telephone No.

Facsimile No.

Teleprinter No.

3. Further observations, if necessary:
Powers of attorney authorizing NOTARBARTOLO & GERVASI SRL to the represent all applicants and applicant/inventors are required.

4. A copy of this notification has been sent to:
☒ the receiving Office
☐ the International Searching Authority
☒ the International Preliminary Examining Authority
☐ the designated Offices concerned
☒ the elected Offices concerned
☒ other: Former agent HOWARD, Paul, Nicholas

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

Authorized officer
M. Abidine
Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

NOTARBARTOLO & GERVASI S.p.A.
Corso di porta Vittoria 9
20122 Milan
ITALIE

Date of mailing (day/month/year)
19 October 1998 (19.10.98)

Applicant's or agent's file reference
1469PTWO

IMPORTANT NOTIFICATION

International application No.
PCT/GB97/01412

International filing date (day/month/year)
23 May 1997 (23.05.97)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address

ISTITUTO NAZIONALE PER LA RICERCA SUL CANCRO
Largo Rosanna Benzi 10
I-16132 Genova
Italy

UNIVERSITA' DI SIENA
Centro Didattico Loc. Le Scotte
I-53100 Siena
Italy

State of Nationality: IT
State of Residence: IT
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [ ] the address [ ] the nationality [ ] the residence

Name and Address

PHILOGEN S.R.L.
Via Roma 22
I-53100 Siena
Italy

State of Nationality: IT
State of Residence: IT
Telephone No.:
Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:

The applicants indicated in Box 1 assigned their rights to the applicant indicatd in Box 2. Therefore the sole applicant is PHILOGEN S.R.L.

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer
Mougamadou Abidine

Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

From the INTERNATIONAL BUREAU

To:

NOTARBARTOLO & GERVASI S.p.A.
Corso di porta Vittoria 9
20122 Milan
ITALIE

Date of mailing (day/month/year) 26 March 1998 (26.03.98)	
Applicant's or agent's file reference 1469PTWO	**IMPORTANT NOTIFICATION**
International application No. PCT/GB97/01412	**International filing date** (day/month/year) 23 May 1997 (23.05.97)

1. The following indications appeared on record concerning:
[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address
MEDICAL RESEARCH COUNCIL
20 Park Crescent
London W1N 4AL
United Kingdom

State of Nationality: GB
State of Residence: GB
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:
[X] the person [X] the name [X] the address [X] the nationality [X] the residence

Name and Address
PHILOGEN S.R.L.
Via Roma 22
53100 Siena
Italy

State of Nationality: IT
State of Residence: IT
Telephone No.:
Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:
Please note the change in the agents file reference number

4. A copy of this notification has been sent to:
[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

Authorized officer
A. Addae-Ruesch
Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

NOTARBARTOLO & GERVASI S.p.A.
Corso di porta Vittoria 9
20122 Milan
ITALIE

Date of mailing (day/month/year)
13 February 1998 (13.02.98)

Applicant's or agent's file reference
P17156WO

IMPORTANT NOTIFICATION

International application No.
PCT/GB97/01412

International filing date (day/month/year)
23 May 1997 (23.05.97)

1. The following indications appeared on record concerning:

[ ] the applicant [ ] the inventor [X] the agent [ ] the common representative

Name and Address
NOTARBARTOLO & GERVASI SRL
Via Savoia 82
I-00198 Rome
Italy

State of Nationality:
State of Residence:
Telephone No.:
Facsimile No.:
Teleprinter No.: 267209

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
NOTARBARTOLO & GERVASI S.p.A.
Corso di porta Vittoria 9
20122 Milan
Italy

State of Nationality:
State of Residence:
Telephone No.: 39 2 541799.1
Facsimile No.: 39 2 54179920
Teleprinter No.: 267209

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer
P.Regis

Telephone No.: (41-22) 338.83.38

PCT/GB97/01412

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:

HOWARD, Paul, Nicholas
Carpmaels & Ransford
43 Bloomsbury Square
London WC1A 2RA
ROYAUME-UNI

Date of mailing (day/month/year) 04 December 1997 (04.12.97)	
Applicant's or agent's file reference P17156WO	**IMPORTANT NOTICE**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/GB97/01412	23 May 1997 (23.05.97)	24 May 1996 (24.05.96)

Applicant
MEDICAL RESEARCH COUNCIL et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,BR,CA,CN,EP,IL,JP,KP,KR,NO,PL,SK,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AL,AM,AP,AT,AZ,BA,BB,BG,BY,CH,CU,CZ,DE,DK,EA,EE,ES,FI,GB,GE,GH,HU,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MD,MG,MK,MN,MW,MX,NZ,OA,PT,RO,RU,SD,SE,SG,SI,TJ,TM,TR,TT,UA,UG,UZ,VN,YU
   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   04 December 1997 (04.12.97) under No. WO 97/45544

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer
J. Zahra

Telephone No. (41-22) 338.83.38

Continuation of Form PCT/IB/308

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

Date of mailing (day/month/year) 04 December 1997 (04.12.97)	IMPORTANT NOTICE
Applicant's or agent's file reference P17156WO	**International application No.** PCT/GB97/01412

The applicant is hereby notified that, at the time of establishment of this Notice, the time limit under Rule 46.1 for making amendments under Article 19 has not yet expired and the International Bureau had received neither such amendments nor a declaration that the applicant does not wish to make amendments.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

INFORMATION CONCERNING ELECTED OFFICES NOTIFIED OF THEIR ELECTION

(PCT Rule 61.3)

To:

HOWARD, Paul, Nicholas
Carpmaels & Ransford
43 Bloomsbury Square
London WC1A 2RA
ROYAUME-UNI

Date of mailing (day/month/year) 08 January 1998 (08.01.98)		
Applicant's or agent's file reference P17156WO	**IMPORTANT INFORMATION**	
International application No. PCT/GB97/01412	**International filing date** (day/month/year) 23 May 1997 (23.05.97)	**Priority date** (day/month/year) 24 May 1996 (24.05.96)
Applicant MEDICAL RESEARCH COUNCIL et al		

1. The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Offices of its election:

   AP :GH,KE,LS,MW,SD,SZ,UG
   EP :AT,BE,CH,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE
   National :AU,BG,BR,CA,CN,CZ,DE,FI,GB,IL,JP,KP,KR,MN,NO,NZ,PL,RO,RU,SE,SK,
   US,VN

2. The following Offices have waived the requirement for the notification of their election; the notification will be sent to them by the International Bureau only upon their request:

   EA :AM,AZ,BY,KG,KZ,MD,RU,TJ,TM
   OA :BF,BJ,CF,CG,CI,CM,GA,GN,ML,MR,NE,SN,TD,TG
   National :AL,AM,AT,AZ,BA,BB,BY,CH,CU,DK,EE,ES,GE,GH,HU,IS,KE,KG,KZ,LC,LK,
   LR,LS,LT,LU,LV,MD,MG,MK,MW,MX,PT,SD,SG,SI,TJ,TM,TR,TT,UA,UG,UZ,YU

3. The applicant is reminded that he must enter the "national phase" **before the expiration of 30 months from the priority date** before each of the Offices listed above. This must be done by paying the national fee(s) and furnishing , if prescribed, a translation of the international application (Article 39(1)(a)), as well as, where applicable, by furnishing a translation of any annexes of the international preliminary examination report (Article 36(3)(b) and Rule 74.1).

   Some offices have fixed time limits expiring later than the above-mentioned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide.

   The entry into the European regional phase is postponed **until 31 months from the priority date** for all States designated for the purposes of obtaining a European patent.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer:
M. Abidine

Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

From the **RECEIVING OFFICE**

To:
Carpmaels & Ransford
43 Bloomsbury Square
LONDON

WC1A 2RA

PCT

NOTIFICATION OF THE INTERNATIONAL APPLICATION NUMBER AND OF THE INTERNATIONAL FILING DATE

(PCT Rule 20.5(c))

Date of mailing *(day/month/year)*	- 6 JUN 1997
Applicant's or agents's file reference **P17156WO**	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB97/01412	**23/05/1997**	**24/05/1996**

Applicant
Medical Research Council et al

Title of the invention
Specific binding members, materials and methods

1. The applicant is hereby notified that the international application has been accorded the international application number and the international filing date indicated above.

2. The applicant is further notified that the record copy of the international application: - 6 JUN 1997

☑ was transmitted to the International Bureau on _____.

☐ has not yet been transmitted to the International Bureau for the reason indicated below and a copy of this notification has been sent to the International Bureau*:

☐ because the necessary national security clearance has not yet been obtained.

☐ because *(reason to be specified)*:

* The International Bureau monitors the transmittal of the record copy by the receiving Office and will notify the applicant (with Form PCT/IB/301) of its receipt. Should the record copy not have been received by the expiration of 14 months from the priority date, the International Bureau will notify the applicant (Rule 22.1(c)).

Name and mailing address of the receiving Office The Patent Office Cardiff Road, Newport South Wales NP9 1RH	Authorized officer STEVE BEVAN ROOM :- G/73 EXT :- 6383
Facsimile No.	Telephon No.

Form PCT/RO/105 (July 1992)

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)*

Box No. I TITLE OF INVENTION

SPECIFIC BINDING MEMBERS, MATERIALS AND METHODS

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

MEDICAL RESEARCH COUNCIL
20 PARK CRESCENT
LONDON W1N 4AL
UNITED KINGDOM

☐ This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State *(i.e. country)* of nationality: U.K.

State *(i.e. country)* of residence: U.K.

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

ISTITUTO NAZIONALE PER LA RICERCA SUL CANCRO
LARGO ROSANNA BENZI 10
16132 GENOVA
ITALY

This person is:

☒ applicant only

☐ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: ITALY

State *(i.e. country)* of residence: ITALY

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

HOWARD, PAUL NICHOLAS
CARPMAELS & RANSFORD
43 BLOOMSBURY SQUARE
LONDON WC1A 2RA
UNITED KINGDOM

Telephone No. 0171 242 8692

Facsimile No. 0171 405 4166

Teleprinter No. 267209

☐ Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Continuation of Box No. III FURTHER APPLICANTS AND/OR (FURTHER) INVENTORS

If none of the following sub-boxes is used, this sheet is not to be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

UNIVERSITA' DI SIENA
CENTRO DIDATTICO LOC. LE SCOTTE
53100 SIENA
ITALY

This person is:
- [X] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: ITALY

State *(i.e. country)* of residence: ITALY

This person is applicant for the purposes of: [ ] all designated States [X] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

DARIO NERI
INSTITUT FUR MOLEKULARBIOLOGIE UND BIOPHYSIK
ETH-HOENGGERBERG
CH-8093 ZURICH
SWITZERLAND

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: ITALY

State *(i.e. country)* of residence: SWITZERLAND

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [X] the United States of America only [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

BARBARA CARNEMOLLA
LABORATORIO DI BIOLOGIA CELLULARE
ISTITUTO NAZIONALE PER LA RICERCA SUL CANCRO
LARGO ROSANNA BENZI 10
16132 GENOVA
ITALY

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: ITALY

State *(i.e. country)* of residence: ITALY

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [X] the United States of America only [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

ALESSANDRA LEPRINI
LABORATORIO DI BIOLOGIA CELLULARE
ISTITUTO NAZIONALE PER LA RICERCA SUL CANCRO
LARGO ROSANNA BENZI 10
16132 GENOVA
ITALY

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: ITALY

State *(i.e. country)* of residence: ITALY

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [X] the United States of America only [ ] the States indicated in the Supplemental Box

[X] Further applicants and/or (further) inventors are indicated on another continuation sheet.

Continuation of Box No. III FURTHER APPLICANTS AND/OR (FURTHER) INVENTORS

If none of the following sub-boxes is used, this sheet is not to be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

ENRICA BALZA
LABORATORIO DI BIOLOGIA CELLULARE
ISTITUTO NATIONALE PER LA RICERCA SUL CANCRO
LARGO ROSANNA BENZI10
16132 GENOVA
ITALY

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: ITALY

State *(i.e. country)* of residence: ITALY

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [X] the United States of America only [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

PATRIZIA CASTELLANI
LABORATORIO DI BIOLOGIA CELLULARE
ISTITUTO NATIONALE PER LA RICERCA SUL CANCRO
LARGO ROSANNA BENZI 10
16132 GENOVA
ITALY

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: ITALY

State *(i.e. country)* of residence: ITALY

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [X] the United States of America only [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

ALESSANDRO PINI
DIPARTIMENTO DI BIOLOGIA MOLECOLARE
UNIVERSITA' DI SIENA
CENTRO DIDATTICO LOC. LE SCOTTE
53100 SIENA
ITALY

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: ITALY

State *(i.e. country)* of residence: ITALY

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [X] the United States of America only [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

LUCIANO ZARDI
LABORATORIO DI BIOLOGIA CELLULARE
ISTITUTO NAZIONALE PER LA RICERCA SUL CANCRO
LARGO ROSANNA BENZI 10
16132 GENOVA
ITALY

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: ITALY

State *(i.e. country)* of residence: ITALY

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [X] the United States of America only [ ] the States indicated in the Supplemental Box

[X] Further applicants and/or (further) inventors are indicated on another continuation sheet.

See Notes to the request form

Continuation of Box No. III FURTHER APPLICANTS AND/OR (FURTHER) INVENTORS

If none of the following sub-boxes is used, this sheet is not to be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

GREG WINTER
CAMBRIDGE CENTRE FOR PROTEIN ENGINEERING
MRC CENTRE
HILLS ROAD
CAMBRIDGE CB2 2QH
UNITED KINGDOM

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: U.K.

State *(i.e. country)* of residence: U.K.

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

PAOLO NERI
DIPARTIMENTO DI BIOLOGIA MOLECOLARE
UNIVERSITA DI SIENA
CENTRO DIDATTICO LOC. LE SCOTTE
53100 SIENA
ITALY

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: ITALY

State *(i.e. country)* of residence: ITALY

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

This person is:
- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality:

State *(i.e. country)* of residence:

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [ ] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

This person is:
- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality:

State *(i.e. country)* of residence:

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [ ] the United States of America only
- [ ] the States indicated in the Supplemental Box

- [ ] Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

- [X] **AP** **ARIPO Patent:** **KE** Kenya, **LS** Lesotho, **MW** Malawi, **SD** Sudan, **SZ** Swaziland, **UG** Uganda, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- [X] **EA** **Eurasian Patent:** **AM** Armenia, **AZ** Azerbaijan, **BY** Belarus, **KG** Kyrgyzstan, **KZ** Kazakstan, **MD** Republic of Moldova, **RU** Russian Federation, **TJ** Tajikistan, **TM** Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- [X] **EP** **European Patent:** **AT** Austria, **BE** Belgium, **CH and LI** Switzerland and Liechtenstein, **DE** Germany, **DK** Denmark, **ES** Spain, **FI** Finland, **FR** France, **GB** United Kingdom, **GR** Greece, **IE** Ireland, **IT** Italy, **LU** Luxembourg, **MC** Monaco, **NL** Netherlands, **PT** Portugal, **SE** Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- [X] **OA** **OAPI Patent:** **BF** Burkina Faso, **BJ** Benin, **CF** Central African Republic, **CG** Congo, **CI** Côte d'Ivoire, **CM** Cameroon, **GA** Gabon, **GN** Guinea, **ML** Mali, **MR** Mauritania, **NE** Niger, **SN** Senegal, **TD** Chad, **TG** Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

- [X] **AL** Albania .....
- [X] **AM** Armenia .....
- [X] **AT** Austria .....
- [X] **AU** Australia .....
- [X] **AZ** Azerbaijan
- [X] **BA** Bosnia and Herzegovina
- [X] **BB** Barbados
- [X] **BG** Bulgaria .....
- [X] **BR** Brazil .....
- [X] **BY** Belarus
- [X] **CA** Canada
- [X] **CH and LI** Switzerland and Liechtenstein
- [X] **CN** China .....
- [X] **CU** Cuba .....
- [X] **CZ** Czech Republic .....
- [X] **DE** Germany .....
- [X] **DK** Denmark .....
- [X] **EE** Estonia .....
- [X] **ES** Spain .....
- [X] **FI** Finland .....
- [X] **GB** United Kingdom
- [X] **GE** Georgia .....
- [X] **HU** Hungary .....
- [X] **IL** Israel .....
- [X] **IS** Iceland
- [X] **JP** Japan .....
- [X] **KE** Kenya .....
- [X] **KG** Kyrgyzstan .....
- [X] **KP** Democratic People's Republic of Korea .....
- [X] **KR** Republic of Korea .....
- [X] **KZ** Kazakstan .....
- [X] **LC** Saint Lucia
- [X] **LK** Sri Lanka
- [X] **LR** Liberia
- [X] **LS** Lesotho .....
- [X] **LT** Lithuania
- [X] **LU** Luxembourg .....
- [X] **LV** Latvia
- [X] **MD** Republic of Moldova .....
- [X] **MG** Madagascar .....
- [X] **MK** The former Yugoslav Republic of Macedonia .....
- [X] **MN** Mongolia
- [X] **MW** Malawi .....
- [X] **MX** Mexico .....
- [X] **NO** Norway
- [X] **NZ** New Zealand .....
- [X] **PL** Poland .....
- [X] **PT** Portugal .....
- [X] **RO** Romania
- [X] **RU** Russian Federation .....
- [X] **SD** Sudan
- [X] **SE** Sweden
- [X] **SG** Singapore
- [X] **SI** Slovenia .....
- [X] **SK** Slovakia .....
- [X] **TJ** Tajikistan .....
- [X] **TM** Turkmenistan .....
- [X] **TR** Turkey .....
- [X] **TT** Trinidad and Tobago .....
- [X] **UA** Ukraine .....
- [X] **UG** Uganda .....
- [X] **US** United States of America .....
- [X] **UZ** Uzbekistan .....
- [X] **VN** Viet Nam .....

Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet:

- [X] **GH GHANA** .....
- [X] **YU YUGOSLAVIA** .....
- [ ] .....
- [ ] .....

In addition to the designations made above, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except the designation(s) of _____.

The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

Box No. VI PRIORITY CLAIM Further priority claims are indicated in the Supplemental Box ☐

The priority of the following earlier application(s) is hereby claimed:

Country *(in which, or for which, the application was filed)*	Filing Date *(day/month/year)*	Application No.	Office of filing *(only for regional or international application)*
item (1) UNITED KINGDOM	24TH MAY 1996	9610967.3	
item (2)			
item (3)			

Mark the following check-box if the certified copy of the earlier application is to be issued by the Office which for the purposes of the present international application is the receiving Office (a fee may be required):

☒ The receiving Office is hereby requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s) : 9610967.3 (1 COPY)

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(If two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):* ISA /

Earlier search *Fill in where a search (international, international-type or other) by the International Searching Authority has already been carried out or requested and the Authority is now requested to base the international search, to the extent possible, on the results of that earlier search. Identify such search or request either by reference to the relevant application (or the translation thereof) or by reference to the search request:*

Country (or regional Office): Date *(day/month/year)*: Number:

Box No. VIII CHECK LIST

This international application contains the following number of sheets:

1. request	6	sheets
2. description	42	sheets
3. claims	4	sheets
4. abstract	1	sheets
5. drawings	6	sheets
Total	59	sheets

This international application is accompanied by the item(s) marked below:

1. ☐ separate signed power of attorney
2. ☐ copy of general power of attorney
3. ☐ statement explaining lack of signature
4. ☐ priority document(s) *identified in Box No. VI as item(s):*
5. ☒ fee calculation sheet
6. ☐ separate indications concerning deposited microorganisms
7. ☐ nucleotide and/or amino acid sequence listing (diskette)
8. ☒ other *(specify)*: 23|77

Figure No. _____ of the drawings (if any) should accompany the abstract when it is published.

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

MERCER, CHRISTOPHER P.

for HOWARD, PAUL NICHOLAS

For receiving Office use only

1. Date of actual receipt of the purported international application:
2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority specified by the applicant: ISA /
6. ☐ Transmittal of search copy delayed until search fee is paid

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

ATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Articl 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
P 17156WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 97/01412	23/05/1997	24/05/1996
Applicant		
MEDICAL RESEARCH COUNCIL et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [ ] **Certain claims were found unsearchable** (see Box I).

2. [ ] **Unity of invention is lacking** (see Box II).

3. [ ] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing
   - [ ] filed with the international application.
   - [ ] furnished by the applicant separately from the international application,
     - [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
   - [ ] Transcribed by this Authority

4. With regard to the **title,**
   - [ ] the text is approved as submitted by the applicant.
   - [X] the text has been established by this Authority to read as follows:

   ANTIBODIES TO THE ED-B DOMAIN OF FIBRONECTIN, THEIR CONSTRUCTION AND USES

5. With regard to the **abstract,**
   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International Search Report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:

   Figure No. _____
   - [ ] as suggested by the applicant.
   - [ ] because the applicant failed to suggest a figure.
   - [ ] because this figure better characterizes the invention.

   [X] None of the figures.

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

NOTARBARTOLO & GERVASI S.P.A.
Corso di Porta Vittoria, 9
20122 Milano
ITALIE

NOTARBARTOLO & GERVASI
MILANO
RECEIVED
24 AGO. 1998

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of mailing *(day/month/year)*	19.08.98
Applicant's or agent's file reference: 1469PTWO	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB97/01412	23/05/1997	24/05/1996

Applicant
PHILOGEN S.R.L. et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices. see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0. Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Peralt Lappas, R Tel. (+49-89) 2399-8052

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (PCT/IPEA/416)
1469PTWO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB97/01412	23/05/1997	24/05/1996
International Patent Classification (IPC) or national classification and IPC		
C12N15/13		
Applicant		
PHILOGEN S.R.L. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

I	☒	Basis of the report
II	☒	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain documents cited
VII	☐	Certain defects in the international application
VIII	☒	Certain observations on the international application

Date of submission of the demand	Date of completion of this report
11/12/1997	19.08.98
Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0. Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Julia. P Telephone No. (+49-89) 2399-8410

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB97/01412

I. Basis of th r port

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-52 as originally filed

Claims, No.:

1-29 as originally filed

Drawings, sheets:

1/9-9/9 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

s e separate sheet

V. R asoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	3, 9-22, 24-28
	No:	Claims	1-2, 4-8, 23, 29
Inventive step (IS)	Yes:	Claims	3, 9-17, 19-21, 24-28
	No:	Claims	1-2, 4-8, 18, 22-23, 29
Industrial applicability (IA)	Yes:	Claims	1-29
	No:	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

1. Additional remarks to item II :

This international preliminary examination report (IPER) has been done considering the priority date 24.05.96 as a valid date. If it was not so the document B. Carnemolla et al., Int. J. Cancer 1996, Vol. 68 (3), pages 397-405 would become relevant.

2. Additional remarks to item V :

The present application discloses a specific binding member (antibody antigen binding domain) which is specific for and binds directly to the ED-B oncofoetal domain of fibronectin (FN) (B+ fibronectin isoform (B-FN) comprising the ED-B domain), wherein said binding member is exemplified by an antibody having a variable heavy (VH) chain region derived from human germline DP47 (E1-R98 Figure 1) and the CDR3 sequence SLPK or GVGAFRPYRKHE and/or a variable light (VL) chain derived from human germline DPL16 (S1-S90) and the remainder of the CDR3 sequence PVVLNGVV or PFEHNLVV. A pharmaceutical composition comprising said binding member, nucleic acid encoding this binding member, phage that encodes it, transformed or transfected host cells, use in therapy, use in the manufacture of a medicament for the imaging or targeting of tumours as well as a process for production of a corresponding recombinant binding member, process for screening and production of said binding member using an antibody phage library and a diagnostic kit comprising said member are also explicitly claimed.

The attention of the Applicant is drawn to the fact that even if in the description it is said that the claimed binding members **may** be provided as isolates or in a purified form (page 11 lines 11-16), the actual claimed subject matter does not refer to any "isolated" or "purified" binding member and thus, it embraces polyclonal antisera reactive with recombinant ED-B (obtained from polyclonal antibodies to recombinant fusion proteins containing the B+ isoform, such as the one described on page 5 of the application).

The following documents have been cited in the International Search Report (ISR) as being relevant for assessing the novelty and inventiveness of the claimed subject matter:

1) J.H. Peters et al., Cell Adhesion and Communication 1995, Vol. 3, pages 67-89 (**D1**) discloses polyclonal antibodies raised to antigens containing no FN sequences other than the intact ED-B domain and shown to bind **specifically** and **directly** to this domain (page 72). These products and the assay systems disclosed in the document are said to be applicable to immunocytochemical and immunohistologic detection of the ED-B domain

across species as diverse as rat, mouse, human and chicken (page 69, left column, first full paragraph). The fact that the antisera recognises the ED-B domain only after treatment with N-glycanase and that it does not recognise the full-length ED-B produced by mammalian cells is irrelevant for the broadest claims. In view of this disclosure, the Examining Division considers that the subject matter of **claims 1-2, 4-7 and 29** is clearly anticipated by this document and thus, it does not fulfil the requirements of Articles 33 (2) and (3) PCT. In addition, the Examining considers that the subject matter of **claims 18 and 22-23** does not require any inventive contribution from the person skilled in the art and thus, this subject matter does not meet the criteria of Article 33 (3) PCT.

2) D-W. Zang et al., Matrix Biology 1994, Vol. 14 pages 623-633 (**D2**) discloses a polyclonal antibody raised against the canine ED-B domain. Said antibody reacted specifically with canine ED-B and rat ED-B domains (human ED-B domain is also cited as being highly certain). Although deglycosylation is said to be necessary for the exposure of ED-B epitopes on a Western blot assay, it is not necessary in the ELISA assay. In view of this disclosure, the Examining Division considers that the subject matter of **claims 1-2, 4-8 and 29** does not fulfil the requirements of Article 33 (2) and (3) PCT. And as mentioned above, **claims 18 and 22-23** do not require any specific inventive contribution (Article 33 (3) PCT).

3) Documents JP02076598 (**D3**) and JP04169195 (**D4**) refer to two different anti-ED-B monoclonal antibodies which are characterized by their specific ED-B epitope. In view of the actual wording of the claims (result to be achieved, characterization by features of the epitope, etc.. see additional remarks to item VIII), the IPEA considers that these documents also anticipate at least the broad subject matter of **claims 1-2 and 23, 29** (Articles 33 (2) and (3) PCT).

4) EP-A- 0 344 134 (**D5**) discloses the mAb **BC-1**. According to the description of the present application (Table 2 page 30 and page 31 lines 25-29), CGS-1 and CGS-2 only react specifically with FN derivatives that contain the ED-B domain and both have the **same reactivity** as mAb **BC-1** (which also recognizes the B-FN isoform), except in the case of recombinant ED-B, which was not recognised by BC-1. Furthermore, the results shown in the description demonstrate that CGS-1 and CGS-2 bind to ED-B-containing FNs, at regions distinct from one another and distinct from the ED-B structure which is recognised by the mAb BSC-1 (page 33 lines 10-13) but both CGS-1 and CGS-2 reacted

with the **same histological structures** as mAb **BC-1** (qualitatively identical results) (page 34 lines 21-23 and page 35 lines 2-3). There is only one important difference between CGS-1 and CGS-2 and the mAb BC-1, namely that mAb BC-1 is strictly human-specific (page 35 lines 4-9). Even if, **D5** identifies the epitope recognized by mAb BC-1 as being contained in the ED-B domain, it has been subsequently shown not to react with ED-B but instead with an epitope lying within the adjacent constantly expressed seventh FN type III repeat (B. Carnemolla et al., J. Biol. Chem. 1992, Vol. 267 (34), pages 24689-24692 (**D6**)).

Thus, in view of said cited prior art and the broad wording of several claims (see additional remarks to item VIII), the IPEA considers that the subject matter of **claims 1-2, 4-8, 23 and 29** does not fulfil the requirements of Articles 33 (2) and (3) PCT, whereas **claims 18 and 22** do not meet the criteria of Article 33 (3) PCT.

Additional remarks to item VIII :

The following objections are also raised under **Article 6 PCT** concerning the clarity of the claims :

1) According to Article 6 PCT in combination with Rule 6.3 PCT the claims shall define the matter for which protection is sought in terms of technical features. The Examining Division considers that a peptide, polypeptide, protein, oligonucleotide, gene, etc.. being chemical products must be clearly and unambiguously characterized by their amino acid and/or nucleic acid sequences, i.e. by reference to their specific SEQ ID No. The characterization of a product only by the result to be achieved, the desired function or by an arbitrary abbreviation which does not have any real technical meaning is not allowable.

2) Furthermore, the characterization of a product solely by parameters is considered to be exceptional and it should be as a general rule not allowable. It may however be allowable in those cases where the claimed subject matter can not be adequately defined in any other way, provided that these parameters can be clearly and reliably determined and **all** parameters are present in the claim.

3) In this respect, for the characterization of a product in terms of a process of manufacture (product-by-process), it is necessary to identify the structural features or parameters of the product by which the skilled person can **always** and **unequivocally** distinguish the

claimed product from the ones of the prior art, i.e. the process must **always** and **unequivocally** result in a distinguishing feature or parameter. References to the method or process of production of the claimed subject matter ("isolated from synthetic molecular repertoires") are irrelevant if said method does not confer any distinguishing feature in the sense referred above.

Thus, the Examining Division considers that :

a) **claims 1-3 and 15-16** only define the claimed subject matter by the result to be achieved but without disclosing any specific feature of the claimed "specific binding member" which allows to achieve said result (i.e. specific and directly binding to the ED-B oncofoetal domain of fibronectin). A similar objection is raised for the subject matter of **claims 4-8** which only specifies further properties of the domain desired to be bound but not of the "specific binding member" allowing such specific binding.

b) In fact, **claims 1-8 and 15-16** are seen as different "parameters" which intend to characterize the claimed "specific binding member". In agreement with paragraph (1.2) above, all of them should be present in a single claim. However, the claimed subject matter can be more adequately defined (clear and unambiguous) by (all) the specific nucleic acid and/or amino acid sequences given in **claims 9-13**, which in fact are the actual contribution of the present application over the known prior art. (**Claim 13** however refers to a CDR3 sequence which is neither disclosed nor defined in the wording of the claim and thus, the scope of this claim is ambiguous and not clear).

c) the subject matter of **claim 17** is defined by arbitrary abbreviations, namely CGS-1 or CGS-2, without having any technical meaning. General abbreviations such as "ED-B domain" (without any further reference to the corresponding sequence at least in the first independent claim where it is mentioned), B-FN (B+ fibronectin iosoform and corresponding sequence), 7B89, etc.. do not fulfil the requirements of Article 6 PCT in combination with Rule 6.3 PCT.

d) the subject matter of **claim 25** (and dependent **claims 26-27**) refers as an essential feature of the claimed process to a "recombinant antigen" which is however neither disclosed nor defined in the wording of the claim. Thus, this subject matter is not clearly defined.

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(57) Abstract

According to the present invention there is provided a specific binding member which is specific for and binds directly to the ED-B oncofoetal domain of fibronectin (FN). The invention also provides materials and methods for the production of such binding members.

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ANTIBODIES TO THE ED-B DOMAIN OF FIBRONECTIN, THEIR CONSTRUCTION AND USES

Background

This invention relates to specific binding members for a foetal isoform of fibronectin, ED-B, which is also expressed in the developing neovasculature of tumours, as demonstrated both by immunocytochemistry and by targeting of tumours *in vivo*. It also relates to materials and methods relating to such specific binding members.

The primary aim of most existing forms of tumour therapy is to kill as many constituent cells of the tumour as possible. The limited success that has been experienced with chemotherapy and radiotherapy relates to the relative lack of specificity of the treatment and the tendency to toxic side-effects on normal tissues. One way that the tumour selectivity of therapy may be improved is to deliver the agent to the tumour through a binding protein, usually comprising a binding domain of an antibody, with specificity for a marker antigen expressed on the surface of the tumour but absent from normal cells. This form of targeted therapy, loosely termed 'magic bullets', has been mainly exemplified by monoclonal antibodies (mAbs) from rodents which are specific for so-called tumour-associated antigens expressed on the cell surface. Such mAbs may be either chemically conjugated to the cytotoxic moiety (for example, a toxin or a drug) or may be produced as a recombinant fusion protein, where the genes encoding the mAb and the toxin are linked together and expressed in tandem.

The 'magic bullet' approach has had limited, although significant, effect in the treatment of human cancer, most markedly in targeting tumours of lymphoid origin, where the malignant cells are most freely accessible to the therapeutic dose in the circulation. However, the treatment of solid tumours remains a serious clinical problem, in that only a minute proportion of the total cell mass, predominantly the cells at the outermost periphery of the tumour, is exposed to therapeutic immunoconjugates in the circulation; these

peripheral targets form a so-called 'binding site barrier' to the tumour interior (Juweid et al, 1992, *Cancer Res.* **52** 5144-5153). Within the tumour, the tissue architecture is generally too dense with fibrous stroma and closely packed tumour cells to allow the penetration of molecules in the size range of antibodies. Moreover, tumours are known to have an elevated interstitial pressure owing to the lack of lymphatic drainage, which also impedes the influx of exogenous molecules. For a recent review of the factors affecting the uptake of therapeutic agents into tumours, see Jain, R (1994), *Sci. Am.* **271** 58-65.

Although there are obvious limitations to treating solid tumours through the targeting of tumour-associated antigens, these tumours do have a feature in common which provides an alternative antigenic target for antibody therapy. Once they have grown beyond a certain size, tumours are universally dependent upon an independent blood supply for adequate oxygen and nutrients to sustain growth. If this blood supply can be interfered with or occluded, there is realistic potential to starve thousands of tumour cells in the process. As a tumour develops, it undergoes a switch to an angiogenic phenotype, producing a diverse array of angiogenic factors which act upon neighbouring capillary endothelial cells, inducing them to proliferate and migrate. The structure of these newly-formed blood vessels is highly disorganised, with blind endings and fenestrations leading to increased leakiness, in marked contrast to the ordered structure of capillaries in normal tissue. Induction of angiogenesis is accompanied by the upregulation of expression of certain cell surface antigens, many of which are common to the vasculature of normal tissues. Identifying antigens which are unique to neovasculature of tumours has been the main limiting factor in developing a generic treatment for solid tumours through vascular targeting. The antigen which is the subject of the present invention addresses this problem directly.

During tumour progression, the extracellular matrix of the surrounding tissue is remodelled through two main

processes: (1) the proteolytic degradation of extracellular matrix components of normal tissue and (2) the *de novo* synthesis of extracellular matrix components by both tumour cells and by stromal cells activated by tumour-induced cytokines. These two processes, at steady state, generate a 'tumoral extracellular matrix', which provides a more suitable environment for tumour progression and is qualitatively and quantitatively distinct from that of normal tissues. Among the components of this matrix are the large isoforms of tenascin and fibronectin (FN); the description of these proteins as isoforms recognises their extensive structural heterogeneity which is brought about at the transcriptional, post-transcriptional and post-translational level (see below). It is one of the isoforms of fibronectin, the so-called B+ isoform (B-FN), that is the subject of the present invention.

Fibronectins (FN) are multifunctional, high molecular weight glycoprotein constituents of both extracellular matrix and body fluids. They are involved in many different biological processes such as the establishment and maintenance of normal cell morphology, cell migration, haemostasis and thrombosis, wound healing and oncogenic transformation (for reviews see Alitalo et al., 1982; Yamada, 1983; Hynes, 1985; Ruoslahti et al., 1988; Hynes, 1990; Owens et al., 1986). Structural diversity in FNs is brought about by alternative splicing of three regions (ED-A, ED-B and IIICS) of the primary FN transcript (Hynes, 1985; Zardi et al., 1987) to generate at least 20 different isoforms, some of which are differentially expressed in tumour and normal tissue. As well as being regulated in a tissue- and developmentally-specific manner, it is known that the splicing pattern of FN-pre-mRNA is deregulated in transformed cells and in malignancies (Castellani et al., 1986; Borsi et al, 1987; Vartio et al., 1987, Zardi et al, 1987; Barone et al, 1989; Carnemolla et al, 1989; Oyama et al, 1989, 1990; Borsi et al, 1992b). In fact, the FN isoforms containing the ED-A, ED-B and IIICS sequences are expressed to a greater

extent in transformed and malignant tumour cells than in normal cells. In particular, the FN isoform containing the ED-B sequence (B+ isoform), is highly expressed in foetal and tumour tissues as well as during wound healing, but restricted in expression in normal adult tissues (Norton et al, 1987; Schwarzbauer et al, 1987; Gutman and Kornblihtt, 1987; Carnemolla et al, 1989; ffrench-Constant et al, 1989; ffrench-Constant and Hynes, 1989; Laitinen et al, 1991.) B+ FN molecules are undetectable in mature vessels, but upregulated in angiogenic blood vessels in normal (e.g. development of the endometrium), pathologic (e.g. in diabetic retinopathy) and tumour development (Castellani et al, 1994).

The ED-B sequence is a complete type III-homology repeat encoded by a single exon and comprising 91 amino acids. In contrast to the alternatively spliced IIICS isoform, which contains a cell type-specific binding site, the biological function of the A+ and B+ isoforms is still a matter of speculation (Humphries et al., 1986).

The presence of B+ isoform itself constitutes a tumour-induced neoantigen, but in addition, ED-B expression exposes a normally cryptic antigen within the type III repeat 7 (preceding ED-B); since this epitope is not exposed in FN molecules lacking ED-B, it follows that ED-B expression induces the expression of neoantigens both directly and indirectly. This cryptic antigenic site forms the target of a monoclonal antibody (mAb) named BC-1 (Carnemolla et al, 1992). The specificity and biological properties of this mAb have been described in EP 0 344 134 B1 and it is obtainable from the hybridoma deposited at the European Collection of Animal Cell Cultures, Porton Down, Salisbury, UK under the number 88042101. The mAb has been successfully used to localise the angiogenic blood vessels of tumours without crossreactivity to mature vascular endothelium, illustrating the potential of FN isoforms for vascular targeting using antibodies.

However, there remain certain caveats to the specificity of the BC-1 mAb. The fact that BC-1 does not directly

recognize the B+ isoform has raised the question of whether in some tissues, the epitope recognized by BC-1 could be unmasked without the presence of ED-B and therefore lead indirectly to unwanted crossreactivity of BC-1. Furthermore, BC-1 is strictly specific for the human B+ isoform, meaning that studies in animals on the biodistribution and tumour localisation of BC-1 are not possible. Although polyclonal antibodies to recombinant fusion proteins containing the B+ isoform have been produced (Peters et al, 1995), they are only reactive with FN which has been treated with N-glycanase to unmask the epitope.

A further general problem with the use of mouse monoclonal antibodies is the human anti-mouse antibody (HAMA) response (Schroff et al, 1985; Dejager et al, 1988). HAMA responses have a range of effects, from neutralisation of the administered antibody leading to a reduced therapeutic dose, through to allergic responses, serum sickness and renal impairment.

Although polyclonal antisera reactive with recombinant ED-B have been identified (see above), the isolation of mAbs with the same specificity as BC-1 following immunisation of mice has generally proved difficult because human and mouse ED-B proteins show virtually 100% sequence homology. The human protein may therefore look like a self-antigen to the mouse which then does not mount an immune response to it. In fact, in over ten years of intensive research in this field, only a single mAb has been identified with <u>indirect</u> reactivity to the B+ FN isoform (BC-1), with none recognising ED-B <u>directly</u>. It is almost certainly significant that the specificity of BC-1 is for a cryptic epitope exposed as a consequence of ED-B, rather than for part of ED-B itself, which is likely to be absent from mouse FN and therefore not seen as "self" by the immune system of the mouse.

Realisation of the present invention has been achieved using an alternative strategy to those pr viously used and where prior immunisation with fibronectin or ED-B is not required: antibodies with specificity for the ED-B isoform

have been obtained as single chain Fvs (scFvs) from libraries of human antibody variable regions displayed on the surface of filamentous bacteriophage (Nissim et al., 1994; see also WO92/01047, WO92/20791, WO93/06213, WO93/11236, WO93/19172).

We have found using an antibody phage library that specific scFvs can be isolated both by direct selection on recombinant FN-fragments containing the ED-B domain and on recombinant ED-B itself when these antigens are coated onto a solid surface ("panning"). These same sources of antigen have also been successfully used to produce "second generation" scFvs with improved properties relative to the parent clones in a process of "affinity maturation". We have found that the isolated scFvs react strongly and specifically with the B+ isoform of human FN without prior treatment with N-glycanase.

In anti-tumour applications the human antibody antigen binding domains provided by the present invention have the advantage of not being subject to the HAMA response. Furthermore, as exemplified herein, they are useful in immunohistochemical analysis of tumour tissue, both *in vitro* and *in vivo*. These and other uses are discussed further herein and are apparent to the person of ordinary skill in the art.

TERMINOLOGY

Specific binding member

This describes a member of a pair of molecules which have binding specificity for one another. The members of a specific binding pair may be naturally derived or wholly or partially synthetically produced. One member of the pair of molecules has an area on its surface, or a cavity, which specifically binds to and is therefore complementary to a particular spatial and polar organisation of the other member of the pair of molecules. Thus the members of the pair have the property of binding specifically to each other. Examples of types of specific binding pairs are antigen-antibody,

biotin-avidin, hormone-hormone receptor, receptor-ligand, enzyme-substrate.

Antibody

This describes an immunoglobulin whether natural or partly or wholly synthetically produced. The term also covers any polypeptide or protein having a binding domain which is, or is homologous to, an antibody binding domain. These can be derived from natural sources, or they may be partly or wholly synthetically produced. Examples of antibodies are the immunoglobulin isotypes and their isotypic subclasses; fragments which comprise an antigen binding domain such as Fab, scFv, Fv, dAb, Fd; and diabodies.

It is possible to take monoclonal and other antibodies and use techniques of recombinant DNA technology to produce other antibodies or chimeric molecules which retain the specificity of the original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of an antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin. See, for instance, EP-A-184187, GB 2188638A or EP-A-239400. A hybridoma or other cell producing an antibody may be subject to genetic mutation or other changes, which may or may not alter the binding specificity of antibodies produced.

As antibodies can be modified in a number of ways, the term "antibody" should be construed as covering any specific binding member or substance having a binding domain with the required specificity. Thus, this term covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including any polypeptide comprising an immunoglobulin binding domain, whether natural or wholly or partially synthetic. Chimeric molecules comprising an immunoglobulin binding domain, or equivalent, fused to another polypeptide are therefore included. Cloning and expression of chimeric antibodies are described in EP-A-0120694 and EP-A-0125023.

It has been shown that fragments of a whole antibody can

perform the function of binding antigens. Examples of binding fragments are (i) the Fab fragment consisting of VL, VH, CL and CH1 domains; (ii) the Fd fragment consisting of the VH and CH1 domains; (iii) the Fv fragment consisting of the VL and VH domains of a single antibody; (iv) the dAb fragment (Ward et al., 1989) which consists of a VH domain; (v) isolated CDR regions; (vi) F(ab')2 fragments, a bivalent fragment comprising two linked Fab fragments (vii) single chain Fv molecules (scFv), wherein a VH domain and a VL domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site (Bird et al, 1988; Huston et al, 1988) (viii) bispecific single chain Fv dimers (PCT/US92/09965) and (ix) "diabodies", multivalent or multispecific fragments constructed by gene fusion (WO94/13804; Holliger et al, 1993).

Diabodies are multimers of polypeptides, each polypeptide comprising a first domain comprising a binding region of an immunoglobulin light chain and a second domain comprising a binding region of an immunoglobulin heavy chain, the two domains being linked (e.g. by a peptide linker) but unable to associate with each other to form an antigen binding site: antigen binding sites are formed by the association of the first domain of one polypeptide within the multimer with the second domain of another polypeptide within the multimer (WO94/13804).

Where bispecific antibodies are to be used, these may be conventional bispecific antibodies, which can be manufactured in a variety of ways (Holliger and Winter, 1993), eg prepared chemically or from hybrid hybridomas, or may be any of the bispecific antibody fragments mentioned above. It may be preferable to use scFv dimers or diabodies rather than whole antibodies. Diabodies and scFv can be constructed without an Fc region, using only variable domains, potentially reducing the effects of anti-idiotypic reaction. Other forms of bispecific antibodies include the single chain "Janusins" described in Traunecker et al, (1991).

Bispecific diabodies, as opposed to bispecific whole antibodies, may also be particularly useful because they can be readily constructed and expressed in *E.coli*. Diabodies (and many other polypeptides such as antibody fragments) of appropriate binding specificities can be readily selected using phage display (WO94/13804) from libraries. If one arm of the diabody is to be kept constant, for instance, with a specificity directed against antigen X, then a library can be made where the other arm is varied and an antibody of appropriate specificity selected.

Antigen binding domain

This describes the part of an antibody which comprises the area which specifically binds to and is complementary to part or all of an antigen. Where an antigen is large, an antibody may only bind to a particular part of the antigen, which part is termed an epitope. An antigen binding domain may be provided by one or more antibody variable domains. Preferably, an antigen binding domain comprises an antibody light chain variable region (VL) and an antibody heavy chain variable region (VH).

Specific

This refers to the situation in which one member of a specific binding pair will not show any significant binding to molecules other than its specific binding partner. The term is also applicable where eg an antigen binding domain is specific for a particular epitope which is carried by a number of antigens, in which case the specific binding member carrying the antigen binding domain will be able to bind to the various antigens carrying the epitope.

Functionally equivalent variant form

This refers to a molecule (the variant) which although having structural differences to another mol cule (the parent) retains some significant homology and also at least some of the biological function of the parent molecule, e.g.

the ability to bind a particular antigen or epitope. Variants may be in the form of fragments, derivatives or mutants. A variant, derivative or mutant may be obtained by modification of the parent molecule by the addition, deletion, substitution or insertion of one or more amino acids, or by the linkage of another molecule. These changes may be made at the nucleotide or protein level. For example, the encoded polypeptide may be a Fab fragment which is then linked to an Fc tail from another source. Alternatively, a marker such as an enzyme, flourescein, etc, may be linked.

Summary of the present invention

According to the present invention there is provided a specific binding member which is specific for the ED-B oncofoetal domain of fibronectin (FN).

Specific binding members according to the invention bind the ED-B domain directly. In one embodiment, a specific binding member binds, after treatment of the FN with the protease thermolysin, to a, any or all FN containing ED-B. In a further embodiment a specific binding member binds to a, any or all FN containing type III homology repeats which include the ED-B domain. Known FNs are identified in two papers by Carnemolla et al., 1989; 1992). Reference to "all FNs containing ED-B" may be taken as reference to all FNs identified in those papers as containing ED-B.

The specific binding member preferably binds human ED-B, and preferably B+FN of at least one other species, such as mouse, rat and/or chicken. Preferably, the specific binding pair member is able to bind both human fibronectin ED-B and a non-human fibronectin ED-B, such as that of a mouse, allowing for testing and analysis of the sbp member in an animal model.

Specific binding pair members according to the present invention bind fibronectin ED-B without competing with the publicly available deposited antibody BC-1 discussed elsewhere herein. BC-1 is strictly specific for human B+ isoform. Specific binding pair members according to the

present invention do not bind the same epitope as BC-1.

Binding of a specific binding member according to the present invention to B+FN may be inhibited by the ED-B domain.

In an aspect of the present invention the binding domain has, when measured as a purified monomer, a dissociation constant (Kd) of 6×10^{-8} M or less for ED-B FN.

In an aspect of the present invention the binding domain is reactive with, i.e. able to bind, fibronectin ED-B without prior treatment of the fibronectin ED-B with N-glycanase.

Specific binding pair members according to the present invention may be provided as isolates or in purified form, that is to say in a preparation or formulation free of other specific binding pair members, e.g. antibodies or antibody fragments, or free of other specific binding pair members able to bind fibronectin ED-B. Preferably, the specific binding members according to the present invention are provided in substantially pure form. They may be "monoclonal" in the sense of being from a single clone, rather than being restricted to antibodies obtained using traditional hybridoma technology. As discussed, specific binding pair members according to the present invention may be obtained using bacteriophage display technology and/or expression in recombinant, e.g. bacterial, host cells. There is no prior disclosure of a monoclonal specific binding pair member which directly binds fibronectin ED-B.

Preferably, the specific binding member comprises an antibody. The specific binding member may comprise a polypeptide sequence in the form of an antibody fragment such as single chain Fv (scFv). Other types of antibody fragments may also be utilised such as Fab, Fab', F(ab')2, Fabc, Facb or a diabody (Winter and Milstein, 1991; WO94/13804). The specific binding member may be in the form of a whole antibody. The whole antibody may be in any of the forms of the antibody isotypes eg IgG, IgA, IgD, IgE and IgM and any of the forms of the isotype subclasses eg IgG1 or IgG4.

The antibody may be of any origin, for example, human,

murine, ovine or lapine. Other derivations will be clear to those of skill in the art. Preferably, the antibody is of human origin. By "human" is meant an antibody that is partly or entirely derived from a human cDNA, protein or peptide library. This term includes humanized peptides and proteins of non-human origin that have been modified in order to impart human characteristics to the antibody molecule and so allow the molecule to bypass the defences of the human immune system.

The specific binding member may also be in the form of an engineered antibody e.g. a bispecific antibody molecule (or a fragment such as F(ab')2) which has one antigen binding arm (i.e. specific domain) against fibronectin ED-B as disclosed and another arm against a different specificity, or a bivalent or multivalent molecule.

In addition to antibody sequences, the specific binding member may comprise other amino acids, e.g. forming a peptide or polypeptide, or to impart to the molecule another functional characteristic in addition to ability to bind antigen. For example, the specific binding member may comprise a label, an enzyme or a fragment thereof and so on.

The binding domain may comprise part or all of a VH domain encoded by a germ line segment or a re-arranged gene segment. The binding domain may comprise part or all of a VL kappa domain or a VL lambda domain.

The binding domain may comprise a VH1, VH3 or VH4 germ-line gene sequence, or a re-arranged form thereof.

A specific binding member according to the present invention may comprise a heavy chain variable region ("VH" domain) derived from human germline DP47, the sequence of which is shown in Figure 1(a), residues 1 to 98. The 'DP' nomenclature is described in Tomlinson et al, (1992). The amino acid sequence of the CDR3 may be Ser Leu Pro Lys. The amino acid sequence of the CDR3 may be Gly Val Gly Ala Phe Arg Pro Tyr Arg Lys His Glu. Thus, the binding domain of a specific binding member according to the present invention may include a VH domain that comprises the amino acid

sequences shown in Figure 1(a) for CGS1 and CGS2.

The binding domain may comprise a light chain variable region ("VL" domain) derived from human germline DPL16, the sequence of which is shown in Figure 1(b) as codons 1-90.

The VL domain may comprise a CDR3 sequence Asn Ser Ser Pro Val Val Leu Asn Gly Val Val. The VL domain may comprise a CDR 3 sequence Asn Ser Ser Pro Phe Glu His Asn Leu Val Val.

Specific binding members of the invention may comprise functionally equivalent variants of the sequences shown in Figure 1, e.g. one or more amino acids has been inserted, deleted, substituted or added, provided a property as set out herein is retained. For instance, the CDR3 sequence may be altered, or one or more changes may be made to the framework regions, or the framework may be replaced with another framework region or a modified form, provided the specific binding member binds ED-B.

One or more CDR's from a VL or VH domain of an antigen binding domain of an antibody disclosed herein may be used in so-called "CDR-grafting" in which one or more CDR sequences of a first antibody is placed within a framework of sequences not of that antibody, e.g. of another antibody, as disclosed in EP-B-0239400. CDR sequences for CGS1 and CGS2 are shown in Figure 1(a) and 1(b).

A specific binding member according to the invention may be one which competes with an antibody or scFv described herein for binding to fibronectin ED-B. Competition between binding members may be assayed easily *in vitro*, for example by tagging a specific reporter molecule to one binding member which can be detected in the presence of other untagged binding member(s), to enable identification of specific binding members which bind the same epitope or an overlapping epitope.

A specific binding member according to the present invention may be used in a method comprising causing or allowing binding of the specific binding member to its epitope. Binding may follow administration of the specific binding member to a mammal, e.g. human or rodent such as

mouse.

The present invention provides the use of a specific binding member as above to use as a diagnostic reagent for tumours. Animal model experimental evidence described below shows that binding members according to the present invention are useful in *in vivo* tumour localisation.

Preferred specific binding members according to the present invention include those which bind to human tumours, e.g. in a cryostat section, which show an invasive and angiogenic phenotype and those which bind to embryonic tissues, e.g. in a cryostat section. Binding may be demonstrated by immunocytochemical staining.

In a preferred embodiment, the specific binding member does not bind, or does not bind significantly, tenascin, an extracellular matrix protein.

In another preferred embodiment, the specific binding member does not bind, or does not bind significantly, normal human skin, e.g. in a cryostat section and/or as demonstrated using immunocytochemical staining.

Further embodiments of specific binding members according to the present invention do not bind, or do not bind significantly, to one or more normal tissues (e.g. in cryostat section and/or as demonstrated using immunocytochemical staining) selected from liver, spleen, kidney, stomach, small intestine, large intestine, ovary, uterus, bladder, pancreas, suprarenal glands, skeletal muscle, heart, lung, thyroid and brain.

A specific binding member for ED-B may be used as an *in vivo* targeting agent which may be used to specifically demonstrate the presence and location of tumours expressing or associated with fibronectin ED-B. It may be used as an imaging agent. The present invention provides a method of determining the presence of a cell or tumour expressing or associated with fibronectin ED-B expression, the method comprising contacting cells with a specific binding member as provided and determining the binding of the specific binding member to the cells. The method may be performed *in*

vivo, or *in vitro* on a test sample of cells removed from the body.

The reactivities of antibodies on a cell sample may be determined by any appropriate means. Tagging with individual reporter molecules is one possibility. The reporter molecules may directly or indirectly generate detectable, and preferably measurable, signals. The linkage of reporter molecules may be directly or indirectly, covalently, eg via a peptide bond or non-covalently. Linkage via a peptide bond may be as a result of recombinant expression of a gene fusion encoding antibody and reporter molecule.

One favoured mode is by covalent linkage of each antibody with an individual fluorochrome, phosphor or laser dye with spectrally isolated absorption or emission characteristics. Suitable fluorochromes include fluorescein, rhodamine, phycoerythrin and Texas Red. Suitable chromogenic dyes include diaminobenzidine.

Other reporters include macromolecular colloidal particles or particulate material such as latex beads that are coloured, magnetic or paramagnetic, and biologically or chemically active agents that can directly or indirectly cause detectable signals to be visually observed, electronically detected or otherwise recorded. These molecules may be enzymes which catalyse reactions that develop or change colours or cause changes in electrical properties, for example. They may be molecularly excitable, such that electronic transitions between energy states result in characteristic spectral absorptions or emissions. They may include chemical entities used in conjunction with biosensors. Biotin/avidin or biotin/streptavidin and alkaline phosphatase detection systems may be employed.

The mode of determining binding is not a feature of the present invention and those skilled in the art are able to choose a suitable mode according to their preference and general knowledge.

The signals generated by individual antibody-reporter conjugates may be used to derive quantifiable absolute or

relative data of the relevant antibody binding in cell samples (normal and test). In addition, a general nuclear stain such as propidium iodide may be used to enumerate the total cell population in a sample, allowing the provision of quantitative ratios of individual cell populations relative to the total cells. When a radionucleotide such as ^{125}I, ^{111}In or ^{99m}Tc is attached to an antibody, if that antibody localises preferentially in tumour rather than normal tissues, the presence of radiolabel in tumour tissue can be detected and quantitated using a gamma camera. The quality of the tumour image obtained is directly correlated to the signal:noise ratio.

The antibodies may be utilised as diagnostic agents to trace newly vascularised tumours, and may also be employed, e.g. in modified form, to deliver cytotoxic agents or to trigger coagulation within new blood vessels, thus starving the developing tumour of oxygen and nutrients and constituting an indirect form of tumour therapy.

The present invention also provides for the use of a specific binding member as above to use as a therapeutic reagent, for example when coupled, bound or engineered as a fusion protein to possess an effector function. A specific binding member according to the present invention may be used to target a toxin, radioactivity, T-cells, killer cells or other molecules to a tumour expressing or associated with the antigen of interest.

Accordingly, further aspects of the invention provide methods of treatment comprising administration of a specific binding member as provided, pharmaceutical compositions comprising such a specific binding member, and use of such a specific binding member in the manufacture of a medicament for administration, for example in a method of making a medicament or pharmaceutical composition comprising formulating the specific binding member with a pharmaceutically acceptable excipient.

In accordance with the present invention, compositions provided may be administered to individuals. Administration

is preferably in a "therapeutically effective amount", this being sufficient to show benefit to a patient. Such benefit may be at least amelioration of at least one symptom. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, eg decisions on dosage etc, is within the responsibility of general practioners and other medical doctors. Appropriate doses of antibody are well known in the art; see Ledermann et al., (1991); Bagshawe K.D. et al. (1991).

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may comprise, in addition to active ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. intravenous.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may comprise a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally comprise a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare

suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

A specific binding member according to the present invention may be made by expression from encoding nucleic acid. Nucleic acid encoding any specific binding member as provided itself forms an aspect of the present invention, as does a method of production of the specific binding member which method comprises expression from encoding nucleic acid therefor. Expression may conveniently be achieved by culturing under appropriate conditions recombinant host cells containing the nucleic acid.

The nucleic acid may encode any of the amino acid sequences of the antibody antigen binding domains described herein or any functionally equivalent form. Changes may be made at the nucleotide level by addition, substitution, deletion or insertion of one or more nucleotides, which changes may or may not be reflected at the amino acid level, dependent on the degeneracy of the genetic code.

Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host cells include bacteria, mammalian cells, yeast and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells and many others. A common, preferred bacterial host is *E. coli*.

The expression of antibodies and antibody fragments in prokaryotic cells such as *E. coli* is well established in the art. For a review, see for example Plückthun, (1991). Expression in eukaryotic cells in culture is also available to those skilled in the art as an option for production of a specific binding member, see for recent reviews, for example Reff, (1993); Trill et al. (1995).

Suitable vectors can be chosen or constructed,

containing appropriate regulatory sequences, including promoter sequences, terminator sequences, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. 'phage, or phagemid, as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Short Protocols in Molecular Biology*, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992. The disclosures of Sambrook et al. and Ausubel et al. are incorporated herein by reference.

Thus, a further aspect of the present invention provides a host cell containing nucleic acid as disclosed herein. A still further aspect provides a method comprising introducing such nucleic acid into a host cell. The introduction may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g. vaccinia or, for insect cells, baculovirus. For bacterial cells, suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage.

The introduction may be followed by causing or allowing expression from the nucleic acid, e.g. by culturing host cells under conditions for expression of the gene.

In one embodiment, the nucleic acid of the invention is integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques.

Following production of a specific binding member it may

be used for example in any of the manners disclosed herein, such as in the formulation of a pharmaceutical or a diagnostic product, such as a kit comprising in addition to the specific binding member one or more reagents for determining binding of the member to cells, as discussed.

Further aspects of the invention and embodiments will be apparent to those skilled in the art. In order that the present invention is fully understood, the following examples are provided by way of exemplification only and not by way of limitation. Reference is made to the following figures:

Figure 1 shows aligned amino acid sequences of the VH and VL of scFvs CGS-1 and CGS-2. Figure 1(a) shows VH sequences; Figure 1(b) shows VL sequences. CDRs (1, 2 and 3) are indicated. The most homologous human germline VH to both scFvs is the DP47 segment of the VH3 family; the VL segment of both clones is DPL16, the light chain used to build the original scFv library (Nissim et al, 1994). Residues that distinguish the two clones from each other are underlined.

Figure 2: Figure 2A shows a model of the domain structure of a human FN subunit. The IIICS, ED-A and ED-B regions of variability, due to alternative splicing of the FN pre-mRNA, are indicated. The figure also indicates the internal homologies as well as the main thermolysin digestion products containing ED-B (Zardi et al, 1987). Figure 2B shows 4-18% SDS-PAGE of plasma and WI38VA FN and their thermolysin digests stained with Coomassie Blue and immunoblots probed with BC-1, IST-6, CGS-1 and CGS-2. Undigested (lane 1) and digested plasma FN using thermolysin at 1 μg/mg of FN (lane 3) and 10 μg/mg of FN (lane 4). Undigested (lane 2) and digested WI38VA FN using thermolysin at 1μg/mg (lane 5), 5μg/mg (lane 6) and 10μg/mg (lane 7) of FN. The numbers on the right hand side indicate the main thermolysin digestion products shown in Figure 2A. The values on the left indicate the molecular weight standards in kiloDalton (kD).

Figure 3: Figure 3A shows the FN type III repeat

sequences contained in the fusion and recombinant proteins expressed in E. coli and the reactivity of these proteins with CGS-1 and CGS-2 and with the mAbs BC-1 and IST-6. Figure 3B shows a Coomassie Blue stained gel and alongside the immunoblots probed with CGS-1, CGS-2, BC-1, IST-6. The numbering of the lanes corresponds to that of the peptide constructs in the upper part of the figure. The values on the left indicate the molecular weight standards in kD.

Figure 4: Infrared Mouse Imager; the mouse imager used for the targeting experiments consists of a black, non-fluorescent box equipped with a tungsten halogen lamp, excitation and emission filters specific for the CY7 infrared fluorophore and a computer-controlled 8-bit monochrome CCD-camera.

Figure 5: Targeting of fluorescently labelled antibody fragments to the F9 murine teratocarcinoma using the monomeric scFv(CGS-1) and dimeric scFv(CGS-1)2 directed to B-FN. The dimeric scFv(D1.3)2 with a binding specificity to lysozyme was used as a negative control.

Figure 6: Targeting of fluorescently labelled antibody fragments to the F9 murine teratocarcinoma using the affinity matured scFv(CGS-2) and the lower affinity scFv(28SI) directed to the same epitope of B-FN. Targeting is detected both in a large tumours (approx. 0.6 grams), covered at 48h by a black crust that partially obscures the imaging, and in small tumours (approx. 0.2 grams).

All documents mentioned herein are incorporated by reference.

List of Examples

Example 1 - Isolation of human scFvs specific for the ED-B domain of human FN.

Example 2 - Affinity maturation of human scFvs specific for the ED-B domain of human FN.

Example 3 - Specificity of affinity matured scFvs for ED-B-containing fibronectins.

Example 4 - The use of affinity matured anti-ED-B scFvs in immunocytochemical staining of human and mouse tumour sections.

Example 5 - The use of affinity matured anti-ED-B scFvs in *in vivo* targeting of human tumours.

Example 6 - Targeting of xenografted murine F9 teratocarcinoma in nude mice.

Example 1 - Isolation of human scFvs specific for the ED-B domain of human FN

A human scFv phage library (Nissim et al, 1994) was used for the selection of recombinant antibodies. Two different forms of the ED-B isoform were used as a source of antigen for selection and in both cases, the isoform was recombinant human protein.

Recombinant FN peptides containing the type III repeats 2-11 (B-) and 2-11 (B+) were expressed in *Esherichia coli*.

A construct was made using FN cDNA from the clones pFH154 (Kornblihtt et al 1985), λF10 and λF2 (Carnemolla et al, 1989). The cDNA constructs, spanning bases 2229-4787, (Kornblihtt et al, 1985) were inserted into the vector pQE-3/5 using the QIAexpress kit from Qiagen (Chatsworth, CA). The recombinants FN-III 2-11 (B-) and (B+) were purified by immunoaffinity chromatography using the mAb 3E3 (Pierschbacher et al 1981) conjugated to Sepharose 4B (Pharmacia). DNA fragments for the preparation of the recombinant FN fragments containing the type III homology repeats 7B89, 789, ED-B and FN-6 were produced by polymerase chain reaction (PCR) amplification using UltMa DNA polymerase (Perkin Elmer), using cDNA from clones FN 2-11 (B+) and FN 2-11 (B-) as template. Primers were designed to allow cloning of PCR products into pQE-12 using the QIAexpress kit (Qiagen). They were subsequently transformed into *E. coli* and expressed. All cDNA clones were sequenced using a Sequenase 2.0 DNA sequencing kit (USB).

Recombinant proteins were purified by Ni-NTA chromatography (IMAC), according to the manufacturers'

instructions (Qiagen), using the hexahistidine tag at the carboxy terminus of the FN fragments. The ED-B-βGal fusion protein was prepared by cloning ED-B cDNA into the λgt11 bacteriophage vector, to give clone λED-B. Clone λchFN60 (containing part of the ED-B sequence) was derived as a fusion protein from the cloned chicken FN pchFN60 (Norton et al, 1987).

For the selection of the human scFv phage library, three rounds of panning were performed for each of the two different recombinant antigens (7B89 and ED-B). The antigens were both coated onto immunotubes (Nunc; Maxisorp, Roskilde, Denmark) overnight at 50 μg/ml in PBS (20mM phosphate buffer, 0.15M NaCl, pH 7.2). The first antigen was the recombinant FN fragment 7B89, in which the ED-B domain is flanked by the adjacent type III FN homology repeats; this was coated at 4°C overnight. The second antigen used was recombinant ED-B (Zardi et al, 1987) with a carboxy terminal hexahistidine tag; this protein does not contain lysine residues, so that the terminal amino group of the first amino acid is available for site-specific covalent immobilisation of ED-B to reactive ELISA plates (Nunc; Covalink). Coating was carried out overnight at room temperature.

After three rounds of panning, the eluted phage were infected into HB2151 *E. coli* cells and plated as described (Nissim et al., 1994). After each round of selection, 95 ampicillin-resistant single colonies were screened to identify antigen-specific scFvs by ELISA. Clones which gave the highest ELISA signals on the antigens used for panning were selected for further analysis and for affinity maturation. These clones were also demonstrated to give specific staining of sections of glioblastoma multiforme and breast tumours by immunocytochemical staining, described in more detail in Example 4.

Example 2 - Affinity maturation of human scFvs specific for the ED-B domain of human FN

Clones 35GE (from selection with 7B89) and 28SI (from

selection with the ED-B domain alone) were selected as candidate antibodies for affinity maturation. In order to diversify the light chains as a means of improving affinity, we then explored a simple affinity maturation strategy based on randomising the central six residues (DSSGNH) of the light chain CDR3 using degenerate oligonucleotides and PCR (Fig. 1), providing a potential sequence diversity of $20^6 = 6.4 \times 10^7$. This region (along with the heavy chain CDR3) is located at the centre of the antigen binding site (Padlan, 1994). We also mutated the arginine residue directly preceding the six residue stretch to serine, in order to avoid the possibility of electrostatic effects dominating the selection.

Plasmid from a single bacterial colony expressing the "parent" scFv fragment was PCR amplified with primers LMB3 (5' CAG GAA ACA GCT ATG AC 3') and CDR3-6-VL-FOR (5' CTT GGT CCC TCC GCC GAA TAC CAC MNN MNN MNN MNN MNN MNN AGA GGA GTT ACA GTA ATA GTC AGC CTC 3') (94C [1'] - 55C [1'] - 72C [1'30"], 25 cycles; see Marks et al., 1991, for buffers and conditions) The resulting product was gel-purified (in order to remove traces of the plasmid containing the original scFv gene) and used as template for a second amplification step with primers LMB3 and J1-Not-FOR (5' ATT GCT TTT CCT TTT TGC GGC CGC GCC TAG GAC GGT CAG CTT GGT CCC TCC GCC 3') (94C [1'] - 55C [1'] - 72C [1'30"], 25 cycles). The crude PCR product, which ran as a single band of the correct molecular weight on agarose gel, was directly purified from the PCR mixture using Spin-Bind (FMC, Rockland, ME, USA), double-digested with Nco1/Not1 and ligated into gel-purified Nco1/Not1-digested phagemid pHEN1 (Hoogenboom et al., 1991) containing a dummy Nco1/Not1 insert to facilitate the separation of double-digested from single-digested vector. The vector was prepared with a Qiagen (Chatsworth, CA, U.S.A.) plasmid maxi-prep kit. Approximately 5 μg of digested plamid and of insert were used in the ligation mix, which was extracted once with phenol, once with phenol/choroform/isoamyl alcohol (25:25:1), then ethanol-precipitated using glycogen (Boehringer, Mannheim, Germany) as a carrier and speed-vac dried. The

pellet was resuspended in 20 μl water and electroporated in electrocompetent TG1 E. coli cells (Gibson, 1984). We typically used electrocompetent cells with a titre of 10^9 transformants/μg if glycerol stocks are used, or 10^{10} transformants/μg with freshly-prepared electrocompetent cells. This yielded typically > 10^7 clones with the procedure outlined here.

The maturation library was then processed as for the Nissim library (Nissim et al., 1994) to produce phage particles, which were used for one round of selection on immunotubes using 7B89 (10 μg/ml) as antigen, followed by a round of kinetic selection (Hawkins et al., 1992). This selection step was performed by incubating biotinylated 7B89 (10 nM) with the phage suspension (approx. 10^{12} t.u.) in 2% milk-PBS (2% MPBS) from the first round of selection for 5 minutes, then adding non-biotinylated 7B89 (1 μM) and letting the competition proceed for 30 minutes. 100 μl of streptavidin-coated dynabeads (Dynal: M480) preblocked in 2% MPBS were then added to the reaction mixture, mixed for 2 minutes and then captured on a magnet and washed 10 times with alternate washes of (PBS + 0.1% Tween-20) and PBS. Phage were eluted from the beads with 0.5 ml 100 mM triethylamine. This solution was then neutralised with 0.25 ml 1M Tris, pH 7.4, and used to infect exponentially growing HB2151 cells (Nissim et al., 1994). 95 ampicillin-resistant single colonies were used to produce scFv-containing supernatants (Nissim et al., 1994) which were screened by ELISA, immunohistochemistry and BIAcore to identify the best binders. They were then subcloned between Sfi1/Not1 sites of the pDN268 expression vector (Neri et al., 1996), which appends a phosphorylatable tag, the FLAG epitope and a hexahistidine tag at the C-terminal extremity of the scFv.

Single colonies of the relevant antibodies subcloned in pDN268 were grown at 37°C in 2xTY containing 100 mg/l ampicillin and 0.1% glucose. When the cell culture reached OD^{600} = 0.8, IPTG was added to a final concentration of 1 mM and growth continued for 16-20 hrs at 30°C. After

centrifugation (GS-3 Sorvall rotor, 7000 rpm, 30 minutes), the supernatant was filtered, concentrated and exchanged into loading buffer (50 mM phosphate, pH 7.4 500 mM NaCl, 20 mM imidazole) using a Minisette (Filtron) tangential flow apparatus. The resulting solution was loaded onto 1 ml Ni-NTA resin (Qiagen), washed with 50 ml loading buffer and eluted with elution buffer (50mM phosphate, pH 7.4, 500mM NaCl, 100 mM imidazole). The purified antibody was analysed by SDS-PAGE (Laemmli, 1970) and dialysed versus PBS at 4°C. Purified scFv preparations were further processed by gel-filtration using an FPLC apparatus equipped with a S-75 column (Pharmacia), since it is known that multivalent scFv fragments may exhibit an artificially good binding on BIAcore (Jonsson et al., 1991) by virtue of avidity effects (Nissim et al., 1994; Crothers and Metzger, 1972). The antibody concentration of FPLC-purified monomeric fractions was determined spectrophotometrically assuming an absorbance at 280 nm of 1.4 units for a 1 mg/ml scFv solution.

Binding of monovalent scFv at various concentrations in the 0.1 - 1 μM range in PBS was measured on a BIAcore machine (Pharmacia Biosensor), using the following antigens: (i) 1000 Resonance Units (RU) of biotinylated recombinant FN fragment 7B89 immobilised on a streptavidin coated chip, which was bound specifically by 250 RU of scFv; (ii) 200 RU of recombinant ED-B, chemically immobilised at the N-terminal amino group, which was bound specifically by 600 RU of scFv; (iii) 3500 RU of ED-B-rich fibronectin WI38VA (see Example 3), which was bound specifically by 150 RU of scFv. Kinetic analysis of the data was performed according to the manufacturers' instructions. On the basis of qualitative BIAcore analysis of antibody-containing supernatants, one affinity-matured version of each scFv clone was selected: clone CGS-1 from selection with the 78B9 fragment and CGS-2 from selection with ED-B recombinant FN fragment. The association rate constants (k_{on}) and dissociation rate constants (k_{off}) are shown in Table 1, together with the calculated equilibrium dissociation constants (Kd) of both

scFvs and the original clone 28SI. Although both the CGS-1 and CGS-2 clones have Kds in the nanomolar range, clone CGS-2 showed the best improvement over its parent clone, giving a Kd of 1nM (improved from 110nM) with respect to all three proteins tested on the sensor chip (Table 1). The improvement was due mainly to a slower kinetic dissociation constant (~10^{-4} s^{-1}), as measured with monomeric antibody preparations (not shown).

The maturation strategy appears to be general, and has yielded affinity improved antibodies against maltose binding protein, cytochrome C, the extracellular domain of murine endoglin (D.N., L.Wyder, R. Klemenz), cytomegalovirus (A.P., G. Neri, R. Botti, P.N.), the nuclear tumour marker HMGI-C protein (A.P., P. Soldani, V.Giancotti, P.N.) and the ovarian tumour marker placental alkaline phosphatase (M. Deonarain and A.A. Epenetos). The strategy therefore seems to be at least as effective as other maturation strategies (Marks et al., 1992; Low et al., 1996), and yields antibodies with similar affinities as those derived from very large phage antibody libraries (Griffiths et al., 1994; Vaughan et al.,1996).

The affinity matured clones CGS-1 and CGS-2 were sequenced and aligned to a database of human germline antibody V genes (V-BASE) then translated using MacVector software. The VH gene of both clones was most homologous to human germline DP47 (VH3), and in addition each clone had a different VH CDR3 sequence (Figure 1). The VL gene of both clones was the DPL16 germline used in the construction of the human synthetic scFv repertoire described in Nissim et al, 1994. The VL CDR3 sequences differed from each other at four out of six of the randomised residues (Figure 1b).

TABLE I

Kinetic and dissociation constants of monomeric scFv fragments CGS-1 and CGS-2 towards ED-B domain-containing proteins

Antigen:	ED-B			7B89			FN WI38VA		
ScFv:	CGS-1	SI28	CGS-2	CGS-1	SI28	CGS-2	CGS-1	SI28	CGS-2
k_{off} (s^{-1})*	7.0×10^{-3}	2.7×10^{-2}	1.5×10^{-4}	3.9×10^{-3}	3.0×10^{-2}	2.3×10^{-4}	5.0×10^{-3}	7.1×10^{-2}	6.5×10^{-4}
k_{on} ($M^{-1}s^{-1}$)*	1.3×10^{5}	2.5×10^{5}	1.3×10^{5}	1.1×10^{5}	2.9×10^{5}	1.1×10^{5}	4.1×10^{5}	1.2×10^{6}	2.9×10^{5}
K_d (M)*	5.4×10^{-8}	1.1×10^{-7}	1.1×10^{-9}	3.5×10^{-8}	1.0×10^{-7}	2.1×10^{-9}	1.2×10^{-8}	5.9×10^{-8}	2.4×10^{-9}

Legend to Table I

Experiments were performed as described in the Materials and Methods section.

* The k_{off} and k_{on} values are accurate to +/- 30%, on the basis of the precision of concentration determinations and in relation to the slightly differe results obtained when different regions of the sensograms are used for the fitting procedure. $K_d = k_{off} / k_{on}$.

Example 3 - Specificity of affinity matured scFvs for ED-B-containing fibronectins

The immunoreactivity of the two affinity matured scFvs, CGS-1 and CGS-2, was assessed initially by ELISA and compared directly to the mAb BC-1 (which recognises the B-FN isoform) and mAb IST-6, which only recognises FN isoforms lacking ED-B (Carnemolla et al., 1989; 1992). The characterisation of these mAbs has been previously reported (Carnemolla et al, 1989; 1992). Fine specificity analysis was thereafter carried out using an extensive panel of FN fragments derived by thermolysin treatment and of recombinant fusion proteins.

The antigens used for ELISA and immunoblotting were prepared as follows. FN was purified from human plasma and from the conditioned medium of the WI38VA13 cell line as previously reported (Zardi et al, 1987). Purified FNs were digested with thermolysin (protease type X; Sigma Chemical Co.) as reported by Carnemolla et al (1989). Native FN 110kD (B-) and native FN 120kD (B+) fragments (see Figure 2) were purified from a FN digest as previously reported (Borsi et al, 1991). The large isoform of tenascin-C was purified as reported by Saginati et al (1992). Recombinant proteins were expressed and purified as described in Example 1. SDS-PAGE and Western blotting were carried out as described by Carnemolla et al (1989).

All antigens used in ELISA were diluted in PBS to between 50-100 μg/ml and coated at 4°C overnight onto Immuno-Plate wells (Nunc, Roskilde, Denmark). Unbound antigen was removed with PBS and plates were then blocked with PBS containing 3% (w/v) bovine serum albumin (BSA) for 2h at 37°C. This was followed by four washes with PBS containing 0.05% Tween 20 (PBST). Antibodies were then allowed to bind at 37°C for 1.5h; scFvs were preincubated with an antiserum directed against the tag sequence: mAb M2 [Kodak, New Haven CT] for the FLAG tag or 9E10 [ATCC, Rockville, MD] for the myc tag. Control antibodies tested were mAbs BC-1 and IST-6. After four washes with PBST, the plates were incubated for 1h at 37°C with 1:2000 diluted (in

PBST+3% BSA) biotinylated goat anti-mouse IgG (Bio-SPA Division, Milan, Italy). The washes were repeated and Streptavidin-biotinylated alkaline phosphatase complex (Bio-SPA Division, Milan, Italy) was added (1:800 diluted in PBST containing 2mM MgCl2) for 1h at 37°C. The reaction was developed using Phosphatase substrate tablets (Sigma) in 10% diethanolamine, pH 9.8 and the optical density was read at 405nm. The results are presented below in Table 2.

Table 2

	CGS-1	CGS-2	BC-1	IST-6
Plasma FN	0.07	0.04	0.09	1.73
WI38VA FN	1.16	0.72	1.20	1.12
n110 kD (B-)	0.03	0.01	0.05	1.20
n120 kD (B+)	0.82	0.81	1.20	0.02
rec FN7B89	1.11	1.02	1.02	0.01
rec FN789	0.01	0.01	0.05	1.25
rec ED-B	1.21	1.32	0.15	0.04
rec FN-6	0.01	0.01	0.08	0.03
Tenascin	0.01	0.02	0.06	0.02

Immunoreactivity of scFv and monoclonal antibodies with fibronectin-derived antigens measured by ELISA. The values represent the OD measured at 405nm after subtraction of background signal. The data are the mean of four experiments showing a maximum 10% standard deviation.

The identity of the different forms of fibronectin used in the experiment is as follows: Plasma FN = human plasma fibronectin; WI38-VA FN = fibronectin from supernatants of SV40-transformed fibroblasts (Zardi et al, 1987); n110kD =

thermolysin treated FN domain 4, without ED-B; n120kD = thermolysin treated FN domain 4, containing ED-B; rec FN7B89 = ED-B domain flanked by adjacent type III FN homology repeats; rec FN789 = type III FN homology repeats with an ED-B domain; rec ED-B = recombinant ED-B alone; rec FN6 = recombinant FN domain 6.

Both CGS-1 and CGS-2 recognised the recombinant ED-B peptide, as well as all native or recombinant FN fragments containing the ED-B sequence, while they did not bind to any FN fragments lacking ED-B. Furthermore, CGS-1 and CGS-2 did not react with tenascin (which comprises fifteen type III homology repeats: Siri et al, 1991) and plasma FN, which does not contain detectable levels of the ED-B sequence in thermolysin digestion products (Zardi et al, 1987). In contrast, CGS-1 and CGS-2 reacted strongly with FN purified from the SV40-transformed cell line WI38VA. About 70-90% of FN molecules from this cell line contain ED-B, as shown by thermolysin digestion and S1 nuclease experiments using purified FN and total RNA prepared from the cell line (Zardi et al, 1987; Borsi et al, 1992). The specificity of the scFvs for the ED-B component of FN was demonstrated still further by using soluble recombinant ED-B to inhibit binding of CGS-1 and/or CGS-2 to FN on WI38VA cells (data not shown).

The data confirm that CGS-1 and CGS-2 only react specifically with FN derivatives that contain the ED-B domain. They both show the same reactivity as mAb BC-1, except in the case of recombinant ED-B, which was not recognised by BC-1. The intensity of the ELISA signals obtained relative to the mAb controls reflects the high specificity of the two scFvs for ED-B-containing antigens.

The specificity of CGS-1 and CGS-2 was investigated further on immunoblots using FN from plasma and WI38VA cells, and thermolysin digests thereof. Upon thermolysin digestion, FN from WI38VA cells (the majority of which contains ED-B) generates a 120kD fragment (containing ED-B) and a minor 110kD fragment which lacks ED-B (Figure 2A; Zardi et al,

1987). Further digestion of the 120kD domain generates two fragments: a 85kD fragment which contains almost the entire ED-B sequence at its carboxy terminus, and a 35kD sequence (Figure 2A; Zardi et al, 1987).

On the left hand side of Figure 2B is a Coomassie stained gel of the protein fractions analysed by immunoblotting. Plasma FN (lane 1) and thermolysin digests of the protein (lane 3, containing the 110kD protein, and lane 4, containing digested 110kD protein) were not recognised by CGS-1 and CGS-2. In contrast, ED-B-rich FN from WI38VA cells, both intact (lane 2) and after increasing thermolysin digestion (lanes 5, 6 and 7) was recognised by both scFv fragments. The smallest FN-derived fragment that could be recognised specifically by CGS-1 was the 120kD protein (spanning type III repeats 2-11 inclusive), while CGS-2 was able to recognise the 85kD fragment spanning repeats 2-7 in addition to the N-terminus of ED-B (Figure 2B; Zardi et al, 1987). These results indicate that the two scFvs are reactive to distinct epitopes within the ED-B sequence. The binding of CGS-2 to the 85kD domain indicates that the epitope for this clone lies in the amino terminus of ED-B. In contrast, the loss of CGS-1 binding when the 120kD domain is digested to 85kD demonstrates that it recognises an epitope located more toward the carboxy terminus of the ED-B molecule.

The fine specificity of CGS-1 and CGS-2 was investigated further by immunoblotting using recombinant FN fragments and fusion proteins with or without the ED-B sequence. The FN fusion proteins were prepared as described by Carnemolla et al (1989). The results of these experiments are shown in Figure 3; for the association of the schematic diagram to the structure of the domains of human FN, see Carnemolla et al, 1992. The binding profiles obtained essentially confirmed what had previously been found by ELISA and immunoblots on purified FN and proteolytic cleavage products: CGS-1 and CGS-2 were strongly reactive with ED-B-containing FN fragments (lanes 2 and 4) but showed no reactivity to FN

sequences lacking ED-B (lanes 1 and 3). CGS-1 did not react with either the human (lane 5) or the chicken (lane 6) ED-B fusion protein, while CGS-2 reacted strongly with both fragments (Figure 3). This result may reflect certain conformational constraints of the epitope in ED-B-containing FN recognised by CGS-1; it is possible, for example, that the epitope is sensitive to denaturation or is not presented correctly when fractionated by SDS-PAGE and transferred to a solid support such as nitrocellulose.

Taken together, these results demonstrate that CGS-1 and CGS-2 bind strongly and specifically to ED-B-containing FNs, at regions distinct from one another and distinct from the ED-B structure which is recognised by the mAb BC-1.

Example 4 - The use of affinity matured anti-ED-B scFvs in immunocytochemical staining of human and mouse tumours

CGS-1 and CGS-2 have both been used to immunolocalise ED-B containing FN molecules in various normal and neoplastic human tissues. For normal tissue, skin was chosen, since the B-FN isoform is known to be expressed in macrophages and fibroblasts during cutaneous wound healing (Carnemolla et al, 1989; Brown et al, 1993). The two human tumours selected have previously been analysed for the specificity of staining with anti-fibronectin mAbs: glioblastoma multiforme has been studied in detail because endothelial cells in the vessels of this tumour are in a highly proliferative state with increased angiogenetic processes including the expression of B-FN isoforms (Castellani et al, 1994). Furthermore, studies using a diverse panel of normal, hyperplastic and neoplastic human breast tissues have provided further evidence of a correlation between angiogenesis and B-FN expression (Kaczmarek et al, 1994).

For the experiments described here, the immunohistochemical staining of CGS-1 and CGS-2 has been compared to that of mAb BC-1 (which recognises the B-FN isoform) and other mAbs known to react either to all known FN isoform variants (IST-4) or only to FN isoforms lacking

ED-B (IST-6). The characterisation of all of these control antibodies has been previously reported (Carnemolla et al, 1989; 1992).

Normal and neoplastic tissues were obtained from samples taken during surgery. It has already been established that the preparation and fixation of tissues is critical for accurate and sensitive detection of FN-containing molecules (Castellani et al, 1994). For immunohistochemistry, 5μm thick cryostat sections were air dried and fixed in cold acetone for ten minutes. Immunostaining was performed using a streptavidin-biotin alkaline phosphatase complex staining kit (Bio-SPA Division, Milan, Italy) and naphthol-AS-MX-phosphate and Fast Red TR (Sigma). Gill's haematoxylin was used as a counterstain, followed by mounting in glycergel (Dako, Carpenteria, CA) as reported previously by Castellani et al, 1994. In order to analyse specificity further in experiments where positive staining of tissues was obtained, specificity for ED-B was demonstrated by preincubation of antibodies with the recombinant ED-B domain, followed by detection as previously described.

The results of these experiments overall showed that both CGS-1 and CGS-2 reacted with the same histological structures as mAb BC-1. The staining pattern obtained with skin using CGS-1, CGS-2 and BC-1 reflects the absence of ED-B from the FN expressed in the dermis. In the staining of invasive ductal carcinoma sections, CGS-1, CGS-2 and BC-1 showed a restricted distribution of staining, confined to the border between the neoplastic cells and the stroma. This is consistent with the fact that although total FN is homogeneously distributed throughout the tumour stroma, the expression of B-FN is confined to certain regions, and it is these areas that had previously been successfully localised (in 95% of cases) in invasive ductal carcinoma using mAb BC-1 (Kaczmarek et al, 1994).

Previous findings in the staining of BC-1 of glioblastoma multiforme tumour have been confirmed. Castellani et al (1994) had observed a typical pattern of

staining of glomerular-like vascular structures, and in our experiments, CGS-1 and CGS-2 have been shown to give qualitatively identical results.

There is, however, an important difference between CGS-1 and CGS-2 and the mAb BC-1: the two human scFvs have been demonstrated to bind to both chicken and mouse B-FN, whereas BC-1 is strictly human-specific. CGS-2 reacted with chicken embryos (data not shown) and both CGS-1 and CGS-2 reacted with mouse tumours.

CGS-1 staining of vascular structures on sections of the murine F9 teratocarcinoma has also been shown. In contrast, all normal mouse tissues tested (liver, spleen, kidney, stomach, small intestine, large intestine, ovary, uterus, bladder, pancreas, suprarenal glands, skeletal muscle, heart, lung, thyroid and brain) showed a negative staining reaction with CGS-1 and CGS-2 (data not shown) The structures stained in the F9 teratocarcinoma sections were shown to be ED-B specific by using the recombinant ED-B domain to completely inhibit the staining obtained (data not shown).

Example 5 - The use of affinity matured anti-ED-B scFvs in in vivo targeting of human tumours

The human melanoma cell-line SKMEL-28 was used to develop xenografted tumours in 6-10 weeks old nude mice (Balb-c or MF-1; Harlan UK), by injecting 1 x 10^7 cells/mouse subcutaneously in one flank. Mice bearing tumours were injected in the tail vein with 100 μl of 1 mg/ml $scFv_1$-$Cy7_1$ solution in PBS when tumours had reached a diameter of approximately 1cm.

Labeling of recombinant antibodies with CY7 was achieved by adding 100μl 1M sodium bicarbonate, pH=9.3, and 200μl CY7-bis-OSu (Amersham; Cat. Nr. PA17000; 2mg/ml in DMSO) to 1ml antibody solution in PBS (1mg/ml). After 30 minutes at room temperature, 100μl 1M Tris, pH=7.4, was added to the mixture and the labeled antibody was separated from unreacted dye using disposable PD10 columns (Pharmacia Biotech, Piscataway, NJ, USA) equilibrated with PBS. The eluted green antibody

fractions were concentrated to approximately 1mg/ml using Centricon-10 tubes (Amicon, Beverly, MA, USA). The labeling ratio achieved was generally close to one molecule CY7 : one molecule antibody. This was estimated spectroscopically with 1 cm cuvettes, assuming that a 1mg/ml antibody solution gives an absorption of 1.4 units at 280 nm, that the molar extinction coefficient of CY7 at 747 nm is 200'000 ($M^{-1}cm^{-1}$) and neglecting the CY7 absorption at 280nm. Immunoreactivity of the antibody samples after labeling was confirmed either by band-shift (Neri et al., 1996b), by affinity-chromatography on an antigen column or by BIAcore analysis. Mice were imaged with a home-built mouse-imager at regular time intervals, under anesthaesia by inhalation of an oxygen/fluorothane mixture. Two to eight animals were studied for each sample, in order to ascertain the reproducibility of the results. The procedures were performed according to the UK Project Licence "Tumour Targeting" issued to D. Neri (UK PPL 80/1056).

The infrared mouse-imager was built as a modification of the photodetection system of Folli et al. (1994), that allows the use of the infrared fluorophore CY7. Infrared illumination was chosen in order to obtain better tissue penetration. The fluorescence of CY7 (>760 nm) is invisible to humans and requires the use of a computer-controlled CCD-camera. The mouse-imager consisted of a black-painted, light-tight box, equipped with a 100W tungsten halogen lamp, fitted with a 50mm diametre excitation filtre specifically designed for CY7 (Chroma Corporation, Brattleboro, VT, USA; 673-748nm). The resulting illumination beam is, to a good approximation, homogenous over an area of 5 x 10cm size, in which the mouse was placed for imaging. Fluorescence was detected by an 8-bit monochrome Pulnix CCD-camera, equipped with a C-mount lens and a 50mm emission filtre (Chroma Corporation, Brattleboro, VT, USA; 765-855nm), and interfaced with the ImageDOK system (Kinetic Imaging Ltd., Liverpool, UK). This system consists of a computer, equipped with a fram -grabber and software for the capture and integration

of sequential images. Three sequential images acquired in 50ms each were typically used in the averaging process; this number was kept constant for the series of pictures of one animal, to allow a direct comparison of tumour targeting at different time points. Pictures in TIFF format were then converted to PICT files using the program Graphics Converter, and elaborated using the program MacDraw Pro with a Power Macintosh 7100/66 computer.

A schematic outline of the design of this apparatus is depicted in Figure 4.

These experiments demonstrated that both scFv's localised to the tumour when visualised at a macroscopic level.

Microscopic demonstration of targeting of neovasculature of developing tumours with the two anti-EDB scFvs was detailed as follows.

Nude mice and/or SCID mice bearing either a xenografted SKMEL-28 human melanoma or a mouse F9 teratocarcinoma in one flank, were injected with either unlabeled scFv fragments with the FLAG tag, or biotinylated scFv fragments.

Mice were sacrificed at different time points after injection, tumour and non-tumour sections obtained, which were then stained with conventional immunohistochemistry protocols, using either the anti-FLAG M2 antibody (Kodak, 181) or streptavidin-based detection reagents. Optimal targeting was generally obtained at 12 hours post injection. Both CGS1 and CGS2 were demonstrated to bind the neovasculature of both the xenografted human tumour and the murine teratocarcinoma.

Example 6: Targeting of xenografted murine F9 teratocarcinoma in nude mice.

We developed solid tumours in the flank of nude mice by sub-cutaneous injection of 4 x 10^6murine F9 teratocarcinoma cells. This tumour grows very rapidly in mice, reaching 1cm diametre in approximately one week after injection, and is highly vascularised. To image the targeting of the

antibodies, we used a modification of the photodetection methodology of Folli et al (1994), which allows a kinetic evaluation of tumour targeting and of antibody clearance on the same animal imaged at various time points, as is described in detail above (see Figure 4).

For targeting to the tumour and to facilitate detection of the antibodies, scFv(CGS-1), scFv(CGS-2) and the anti-lysozyme scFv(D1.3) (McCafferty et al., 1990) were appended with a homodimerisation tag (Pack et al., 1993) by subcloning the antibodies in the Sfi1/Not1 sites of the expression vector pGIN50. This vector is a derivative of pDN268 (Neri et al., 1996b), in which the His6 sequence of the tag is replaced by the sequence: GGC LTD TLQ AFT DQL EDE KSA LQT EIA HLL KEK EKL EFI LAA H, which contains a cysteine residue and the amphipatic helix of the Fos protein for the covalent homodimerisation of antibody fragments (Abate et al. 1990). Complete covalent dimerisation was not achieved: approximately 30-50% of the antibody fragments consisted of covalently-linked dimers.

Antibody fragments were purified by affinity-chromatography on columns obtained by coupling hen egg lysozyme (D1.3) or 7B89 (anti-ED-B antibodies; Carnemolla et al., 1996) to CNBr-activated Sepharose (Pharmacia Biotech, Piscataway, NJ, USA). Supernatants were loaded onto the affinity supports, which were then washed with PBS, with PBS + 0.5 M NaCl and eluted with 100 mM Et3N. The antibodies were then dialysed against PBS.

The antibodies were labeled as described above and were then injected in the tail vein of tumour-bearing mice with 100μl of 1mg/ml $scFv_1$-$Cy7_1$ solution in PBS, when the tumours had reached a diameter of approximately 1 cm.

As shown in Figure 5, scFv(CGS-1) localised on the tumour for up to three days, though there was also rapid clearance from the tumour during this period. However there was also some staining of the femur. The targeting performance of CGS-1 to the tumour was dramatically improved by introducing an amphipathic helix containing a cysteine

residue at the C-terminus to promote antibody dimerisation (Pack et al., 1993). Indeed the localisation of the dimeric scFv(CGS-2)$_2$ did not appear to significantly decrease from 24 to 72 hours. By contrast, a negative control (the dimeric antibody scFv(D1.3)2, anti-lysozyme antibody), showed a rapid clearance and no detectable localisation on the tumour or femur.

ScFv(28SI) showed weak tumour targeting at 6 hours (not shown) but none was detectable at 24 hours or later (Figure 6). Affinity maturation led to much improved targeting; thus scFv(CGS-2) targeted small and large F9 tumours efficiently, whether as monomer (Figure 6) or dimer (not shown). After two days, the percent injected dose of antibody per gram of tumour was found to be about 2 for the scFv(CGS-2) monomer and 3-4 for the scFv(CGS-2) dimer. The dose delivered to the tumour by scFv(CGS-2) was also higher than for scFv(CGS-1) (Figures 5 and 6), and correlates with their respective affinities (Table 1). However, both scFv(28SI) and scFv(CGS-2) appear to be prone to proteolytic cleavage and show a high liver uptake (Figure 6), whereas scFv(CGS-1) antibodies were significantly more stable and show lower liver uptake (Figure 5).

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Claims

1. A specific binding member which is specific for and binds directly to the ED-B oncofoetal domain of fibronectin (FN).

2. A specific binding member according to claim 1, which comprises an antibody antigen binding domain.

3. A specific binding member according to claim 2, wherein said antibody antigen binding domain is of human origin.

4. A specific binding member according to any one of claims 1 to 3, which binds to all FNs containing ED-B after treatment of the FN with the protease thermolysin.

5. A specific binding member according to any one of claims 1 to 4, which binds to all recombinant FNs containing type III homology repeats which include the ED-B domain.

6. A specific binding member according to any one of claims 1 to 5 whose binding to B-FN is inhibited by the ED-B domain.

7. A specific binding member according to any one of the preceding claims, which binds to B-FN from human, mouse, rat, chicken and any other species in which the ED-B domain is conserved.

8. A specific binding member according to any one of the preceding claims which binds to B-FN without treatment of the FN with N-glycanase.

9. A specific binding member according to any one of the preceding claims having a variable heavy (VH) chain region of the sequence derived from human germline DP47 (codon 1 Glu - codon 98 arg inclusive in Figure 1) and the CDR3 sequence Ser Leu Pro Lys.

10. A specific binding member according to any one of claims 1 to 8 having a variable heavy (VH) chain region of the sequence derived from human germline DP47 (codon 1 Glu - codon 98 Arg inclusive in Figure 1) and the CDR3 sequence Gly Val Gly Ala Phe Arg Pro Tyr Arg Lys His Glu.

11. A specific binding member according to any one of claims 1 to 8 having a variable light (VL) chain region of the sequence derived from human germline DPL16 (codon 1 Ser - codon 90 Ser inclusive in Figure 1) and the remainder of the CDR3 sequence as Pro Val Val Leu Asn Gly Val Val.

12. A specific binding member according to any one of claims 1 to 8 having a variable light (VL) chain region of the sequence derived from human germline DPL16 (codon 1 Ser - codon 90 Ser inclusive in Figure 1) and the remainder of the CDR3 sequence as Pro Phe Glu His Asn Leu Val Val.

13. A specific binding member according to any one of claims 1 to 8 having a variable heavy (VH) chain region of the sequence derived from human germline DP47 (codon 1 Glu - codon 98 arg inclusive in Figure 1) and the CDR3 sequence.

14. A specific binding member according to any one of the preceding claims which, when measured as a purified monomer, has a dissociation constant (K_d) of 6 x 10^{-8}M or less for ED-B FN.

15. A specific binding member according to any one of the preceding claims, wherein said binding member comprises an scF_v molecule.

16. A specific binding member of any one of the preceding claims, wherein said binding member comprises a dimeric scF_v molecule.

17. A specific binding member of any one of the preceding

claims, wherein said binding member comprises CGS-1 or CGS-2.

18. A pharmaceutical composition comprising a specific binding member according to any one of the preceding claims in an effective amount, in conjunction with a pharmaceutically-acceptable excipient.

19. A nucleic acid that encodes a specific binding member according to any one of claims 1 to 17.

20. A phage that encodes a specific binding member according to any one of claims 1 to 17.

21. A host cell transformed or transfected with a nucleic acid acording to claim 19.

22. A specific binding member according to any one of claims 1 to 17 for use in therapy.

23. The use of a specific binding member according to any one of claims 1 to 17 in the manufacture of a medicament for the imaging or targeting of tumours.

24. A process for the production of a specific binding member according to any one of claims 1 to 17, which process comprises expression of a nucleic acid according to claim 19 in a host cell.

25. A process for the production of a specific binding member according to any one of claims 1 to 17, which process comprises:

a) screening a peptide or protein library expressed in phage with recombinant antigen derived from the fibronectin protein;

b) infecting host bacterial cells with positive clones;

c) subjecting positive phage clones to a process of

affinity maturation;

d) repeating steps a) and b) to select positive phage clones with improved affinity for antigen;

e) infecting host cells with positive clones and purifying antibody molecules from said host cells.

26. The process of claim 25, wherein step a) comprises screening a scF_v phage library with recombinant antigen derived from the fibronectin protein.

27. The process of claim 26, wherein said phage library expresses scF_vs of human origin.

28. The process of claim 23, wherein in step a), the phage clones are screened with recombinant antigens 7B89 or ED-B.

29. A diagnostic kit comprising a specific binding member according to any one of claims 1 to 17 and one or more reagents that allow the determination of the binding of said member to cells.

FIG. 1(a)

		CDR1		CDR2
	10 20 30		40	50 60
CGS1	QVQLVESGGGLVQPGGSLRLSCAVSGFTFS	SYAMS	WVRQAPGKGLEWVS	AISGSGGSTYYADSVKG
CGS2	EVQLVESGGGLVQPGGSLRLSCAASGFTFS	SYAMS	WVRQAPGKGLEWVS	AISGSGGSTYYADSVKG

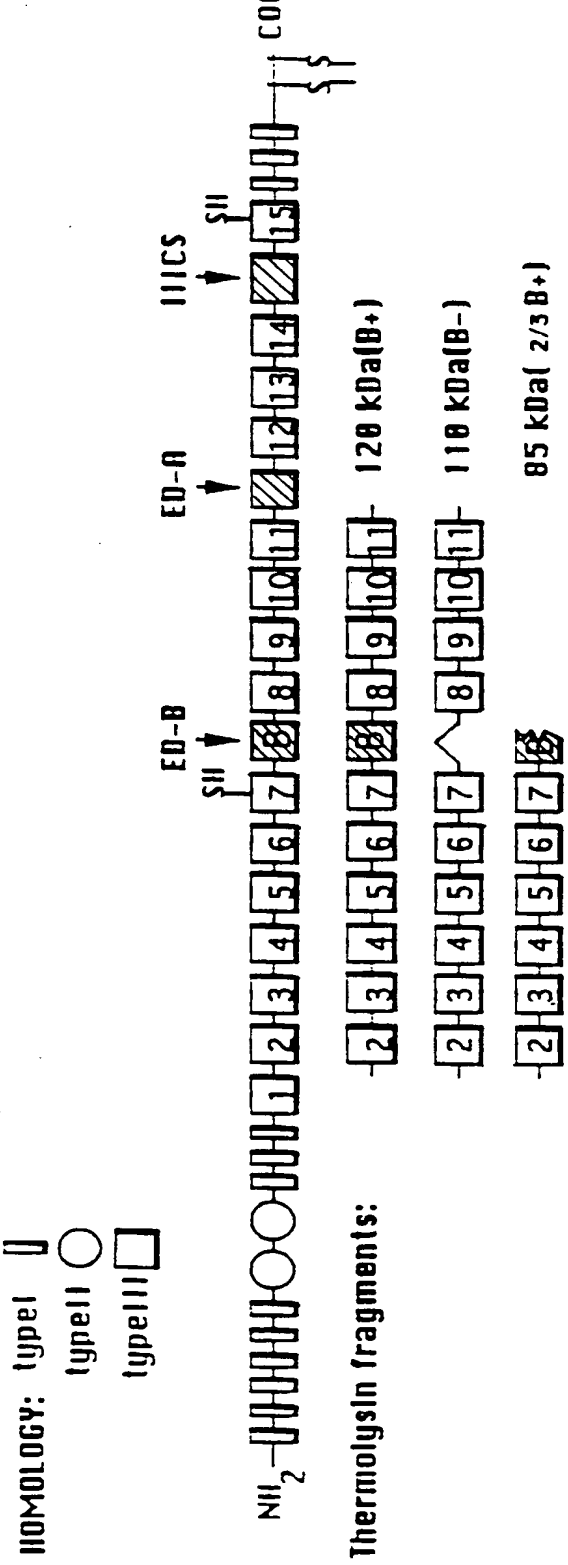
		CDR3	
	70 80 90 98		
CGS1	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR	<u>SLPK</u>	WGQGTLVTVSR
CGS2	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR	<u>GVGAFRPYRKHE</u>	WGQGTLVTVSR

FIG. 1(b)

```
                                   CDR1                                CDR2
            10        20           30          40          50
CGS1  SSELTQDPAVSVALGQTVRITC  QGDSLRSYYAS  WYQQKPGQAPVLVIY  GKNNRPS
CGS2  SSELTQDPAVSVALGQTVRITC  QGDSLRSYYAS  WYQQKPGQAPVLVIY  GKNNRPS

                                                CDR3
         60        70        80                 90          100
CGS1  GIPDRFSGSSSGNTASLTITGAQAEDEADYYC  NSSPVVLNGVV  FGGGTKLTVLG
CGS2  GIPDRFSGSSSGNTASLTITGAQAEDEADYYC  NSSPFEHNLVV  FGGGTKLTVLG
```

FIG. 2A


HOMOLOGY: typeI
typeII
typeIII
ED-B
ED-A
IIICS
S-S
COOH
Thermolysin fragments:
120 kDa(B+)
110 kDa(B-)
85 kDa(2/3 B+)

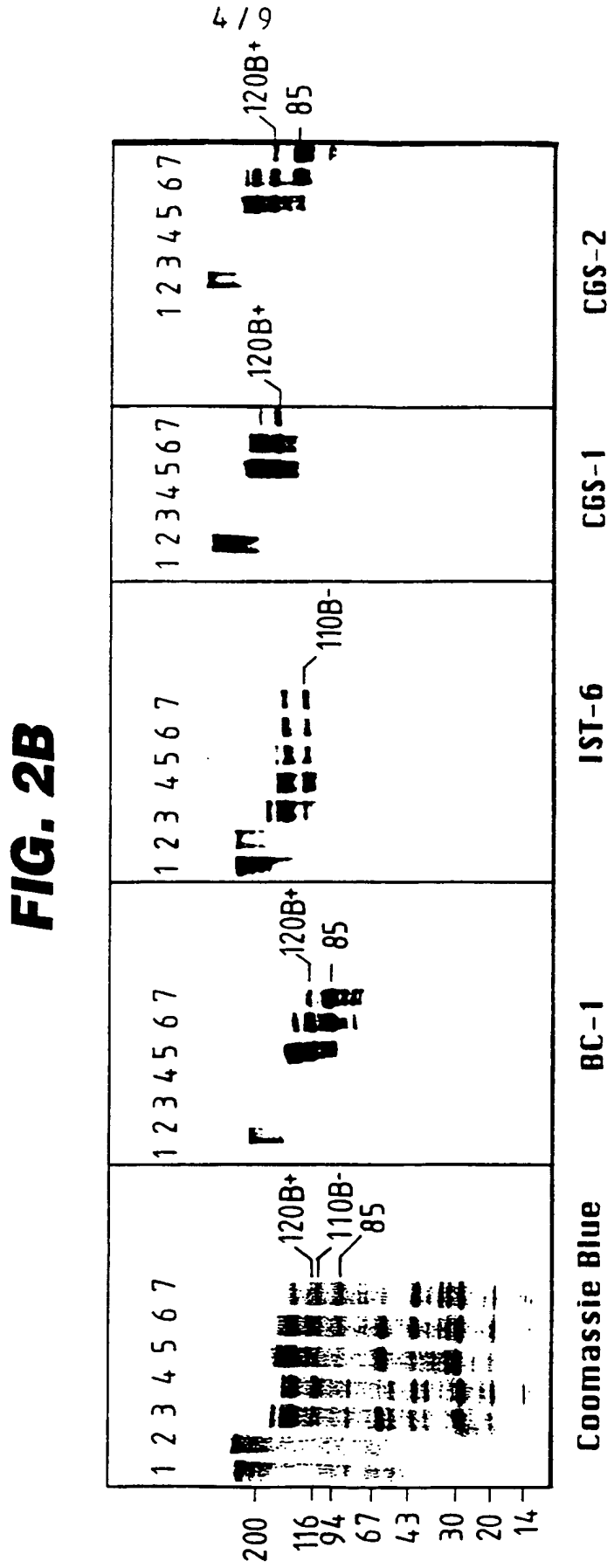
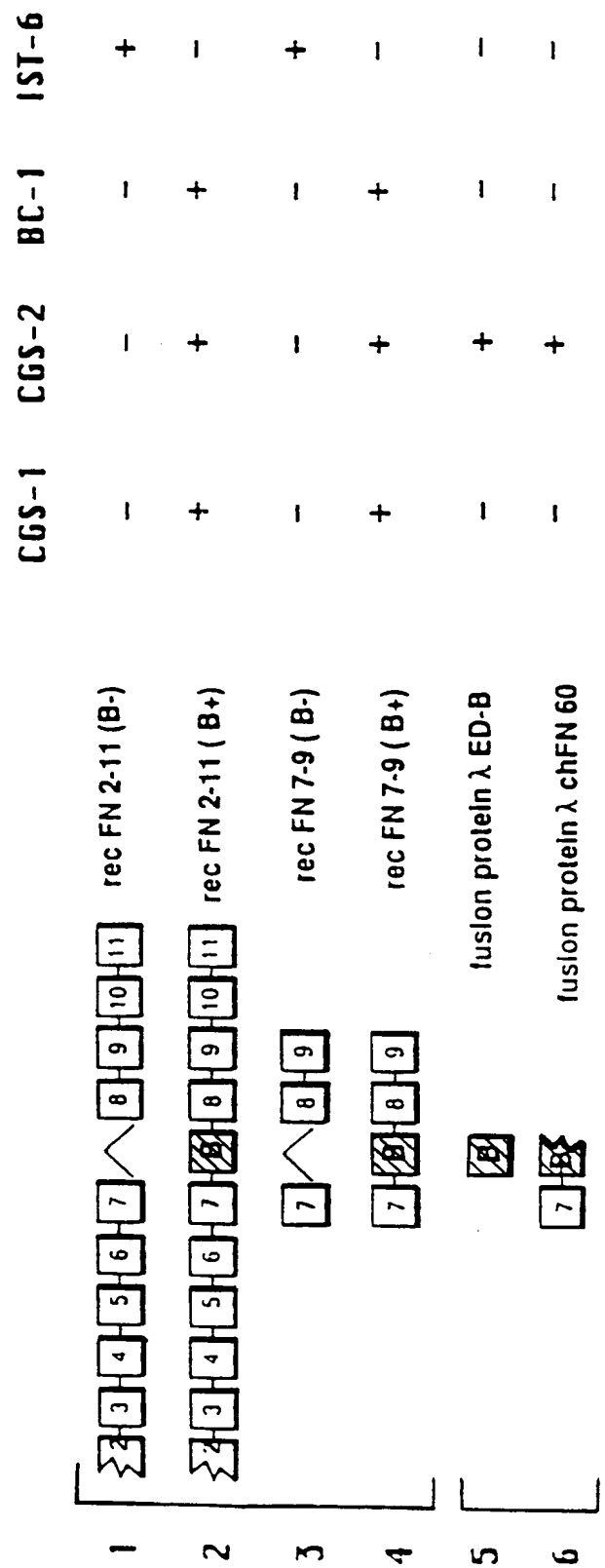

1 2 3 4 5 6 7
120B+
110B-
85
Coomassie Blue
BC-1
IST-6
CGS-1
CGS-2
200
116
94
67
43
30
20
14

FIG. 2B

FIG. 3A


CGS-1
CGS-2
BC-1
IST-6
rec FN 2-11 (B-)
rec FN 2-11 (B+)
rec FN 7-9 (B-)
rec FN 7-9 (B+)
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fusion protein λ chFN 60
1
2
3
4
5
6

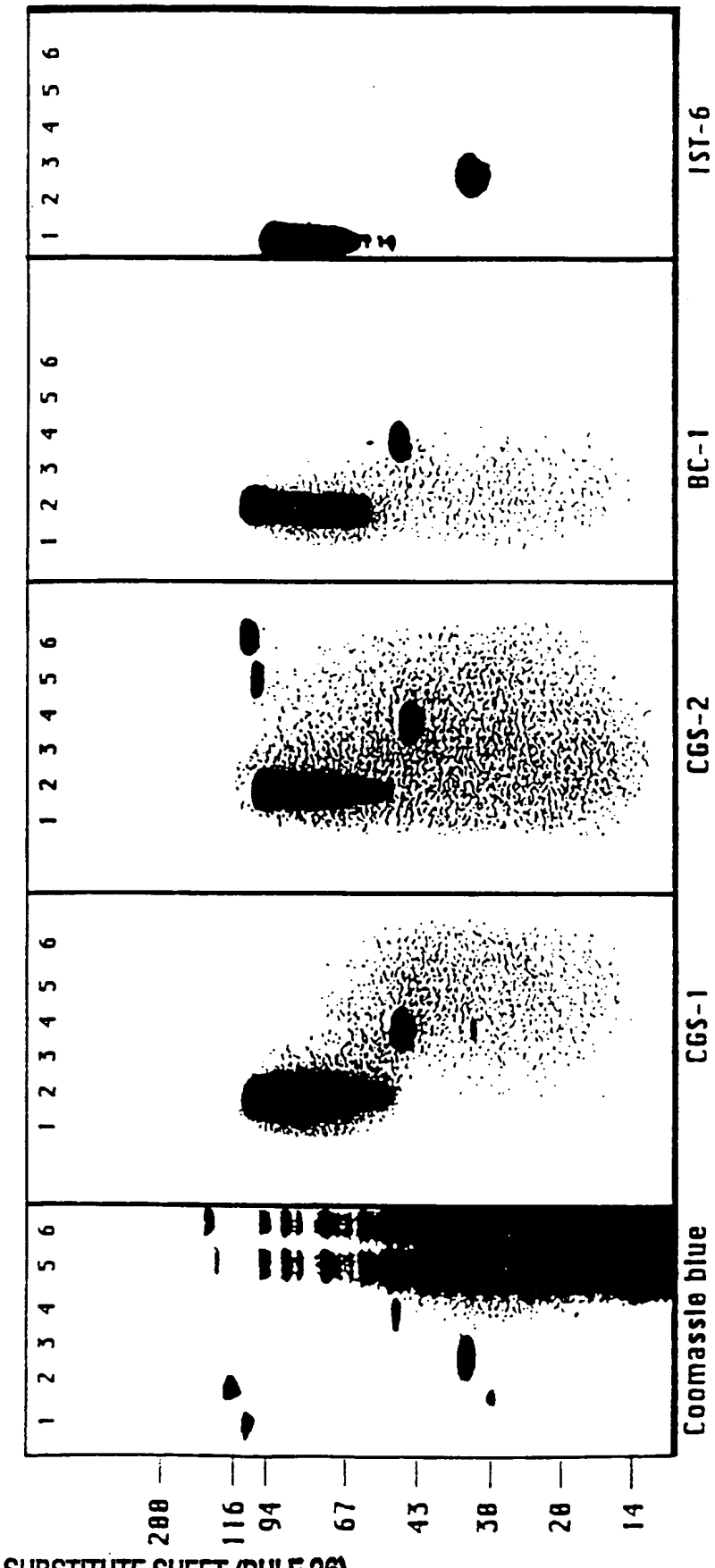

1 2 3 4 5 6
Coomassie blue
CGS-1
CGS-2
BC-1
IST-6
200
116
94
67
43
38
28
14

FIG. 3B

FIG. 4

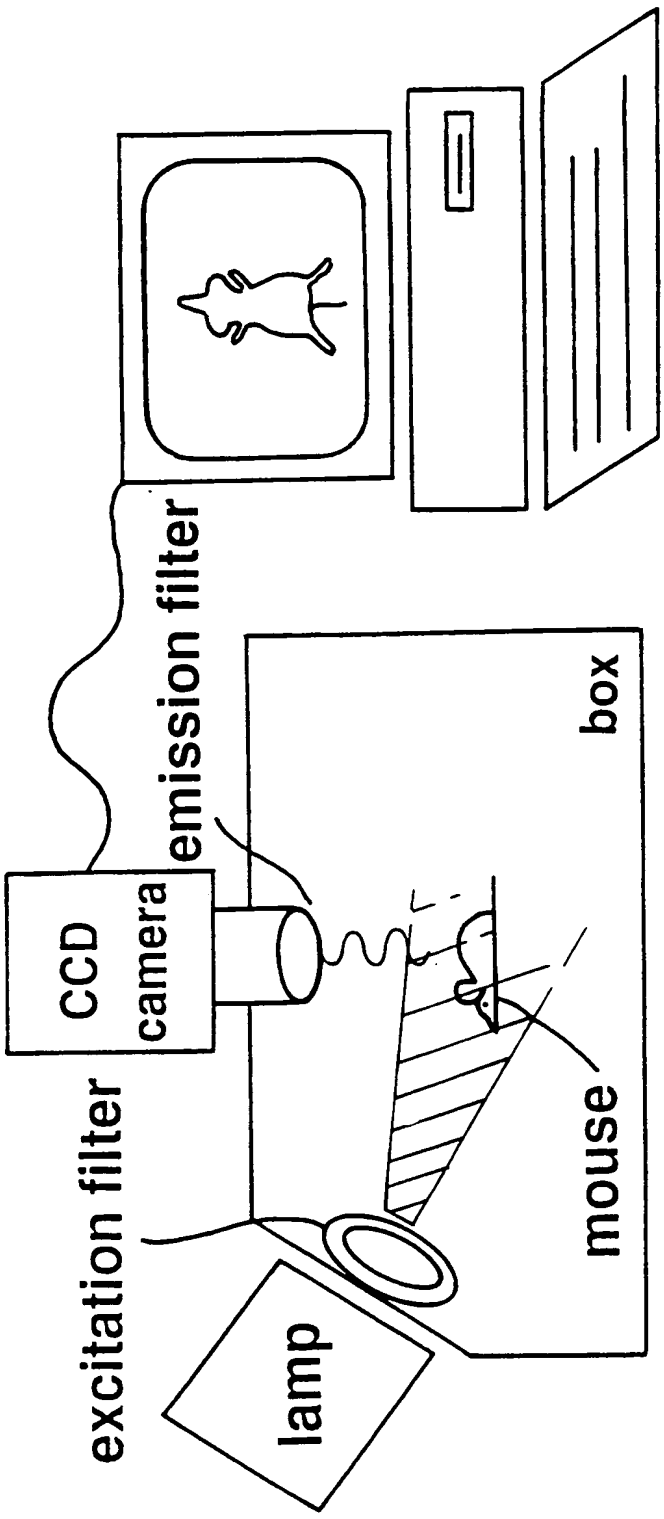
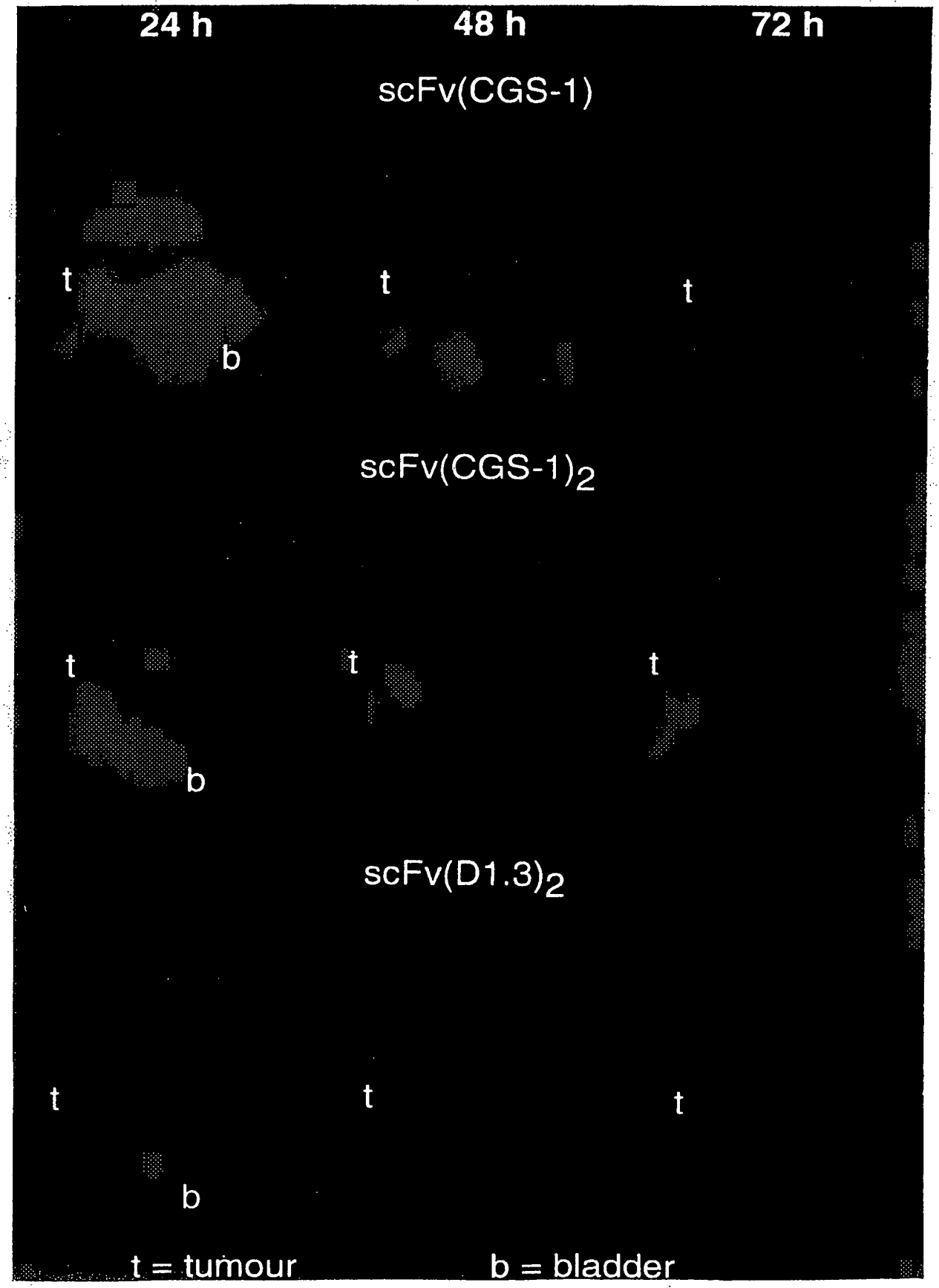

box
emission filter
CCD camera
excitation filter
lamp
mouse

FIG. 5


24 h
48 h
72 h
scFv(CGS-1)
t
b
t
t
scFv(CGS-1)2
t
b
t
t
scFv(D1.3)2
t
b
t
t
t = tumour
b = bladder

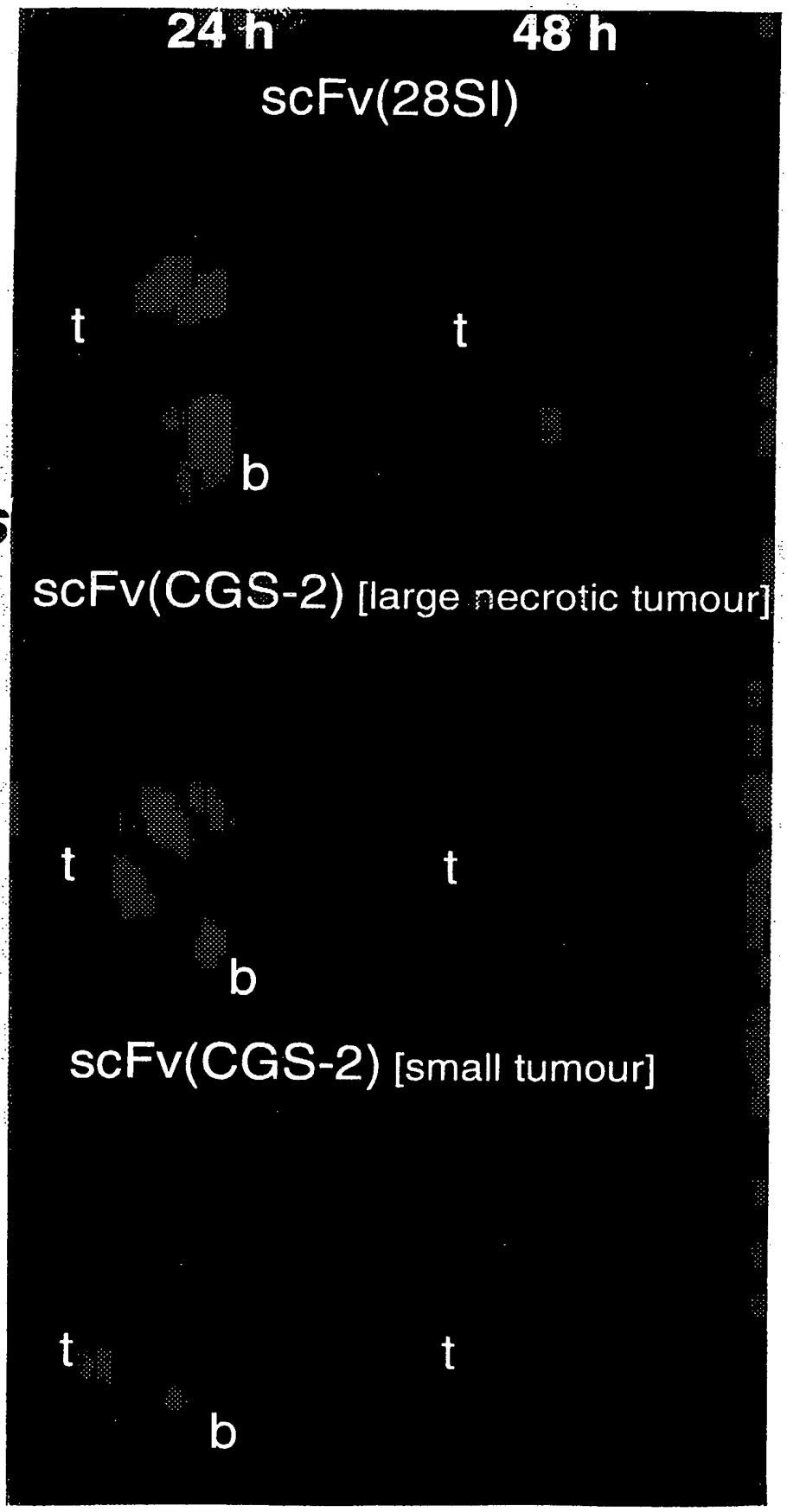

24 h
48 h
scFv(28SI)
t
t
b
scFv(CGS-2) [large necrotic tumour]
t
t
b
scFv(CGS-2) [small tumour]
t
t
b

FIG. 6

INTERNATIONAL SEARCH REPORT

[illegible]tional Application No
PCT/GB 97/01412

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/13 C07K16/18 A61K39/395 C12N1/21 A61K51/10
G01N33/577 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. PETERS ET AL.: "Expression of the alternatively spliced EIIIB segment of fibronectin." CELL ADHESION AND COMMUNICATION, vol. 3, no. 1, 1995, USA, pages 67-89, XP002042097 cited in the application see page 69, left-hand column, line 5 - line 28 see page 72, left-hand column, line 9 - line 42 --- -/--	1,2,5,7, 29

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
29 September 1997	22. 10. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nooij, F

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	D. ZANG ET AL.: "Antibody specific for extra domain B of fibronectin demonstrates elevated levels of both extra domain B(+) and B(-) fibronectin in osteoarthritic canine cartilage." MATRIX BIOLOGY, vol. 14, no. 8, October 1995, STUTTGART, GERMANY, pages 623-633, XP002042098 see the whole document	1,2,5,7, 8,29
A	DATABASE WPI Week 9017 Derwent Publications Ltd., London, GB; AN 90-128252 XP002042103 & JP 02 076 598 A (FUJITA GAKUEN ET AL.) , 15 March 1990 see abstract	1,2,29
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A	US 5 243 029 A (MATSUURA ET AL.) 7 September 1993 see the whole document	1,2,29
A	EP 0 344 134 A (ISTITUTO NAZIONALE PER LA RICERCA SUL CANCRO) 29 November 1989 cited in the application see the whole document	1,2,29
A	P. CASTELLANI ET AL.: "The fibronectin isoform containing the ED-B oncofetal domain: a marker of angiogenesis." INTERNATIONAL JOURNAL OF CANCER, vol. 59, no. 5, 1 December 1994, NEW YORK, NY, USA, pages 612-618, XP002042099 cited in the application see the whole document	1,2,29

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	L. ZARDI ET AL.: "Transformed human cells produce a new fibronectin isoform by preferential alternative splicing of a previously unobserved exon." THE EMBO JOURNAL, vol. 6, no. 8, August 1987, CAMBRIDGE, GB, pages 2337-2342, XP002042100 cited in the application see abstract see figures 1,2 ---	1,2,4,29
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P,X	B. CARNEMOLLA ET AL.: "Phage antibodies with pan-species recognition of the oncofoetal angiogenesis marker fibronectin ED-B domain." INTERNATIONAL JOURNAL OF CANCER, vol. 68, no. 3, 4 November 1996, NEW YORK, NY, USA, pages 397-405, XP002042102 see the whole document -----	1-29

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 97/01412

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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EP 344134 A	29-11-89	DE 68912403 D	03-03-94
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